THE ROLE OF MELANOPSIN IN THE REGULATION OF CIRCADIAN BEHAVIOR AND LIGHT RESPONSES UNDER A WIDE VARIETY OF LIGHT-DARK ENVIRONMENTS

By

Melissa Elizabeth Simmonds

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ABSTRACT

Circadian photoentrainment, the alignment of biological rhythms with the light-dark environment, is necessary for proper physiological functions in virtually all organisms. Light is the strongest regulator of circadian rhythms, especially in mammals. There is still much to learn however, about how light regulates circadian rhythms. During my Ph.D. candidacy, I studied the effects of light on circadian rhythms and on the master biological clock, the suprachiasmatic nucleus (SCN), by using genetically modified mouse lines. More specifically, I studied the contribution of melanopsin, a light responsive protein in the retina, to circadian light responses.

Light exposure at night is known to shift the phase of the circadian clock, as well as induce neural activity in the SCN. In chapter 2, I present and discuss my findings that light differentially activates cells in the SCN in a time-of-day (circa-dian) –dependent manner and found that melanopsin is especially important for SCN light responses during the early night.

Irregular light schedules and extreme light-dark environments can lead to stress-related responses. Responses and recovery of circadian rhythms from the exposure irregular light schedules is discussed in chapter 3. Melanopsin plays a surprising role in these light responses.

In chapter 4, I present my findings that both melanopsin and day length play important roles in circadian photoentrainment, especially when the light-dark environment changes as happens during trans-meridian travel. During these studies, I uncovered an unappreciated role for the acute effect of light on activity (masking) in re-adjusting circadian rhythms to these changes in light-dark environment. My work also uncovered the strong possibility that brain regions other than the SCN play an important role in circadian photoentrainment and in acute light response.

Advisor: Samer Hattar, Ph. D.

Second Reader: Stewart Hendry, Ph. D.

Committee Members: Stewart Hendry, Ph. D.
Nicholas Marsh-Armstrong, Ph. D.
Haiqing Zhao, Ph. D.

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PREFACE

I have always had a burning desire to learn the inner workings of the natural world in which I lived. In particular, I was very curious about how my body functioned. To this end, I decided to study biology in college and possibly become a medical doctor one day. While in college, I realized how little was known about the human body and I was left hungry for more knowledge. Yes, there were many amazing advances in science; however, I found that there were still so many unanswered questions. Therefore, I realized that instead of practicing science in the form of medicine, I needed to help fuel the advancement of new scientific discoveries.

Growing up in the island country of Jamaica, I learned that "No man is an island, no man stands alone" –Joan Baez; therefore, I will take this opportunity to acknowledge some of the key persons in my life who have contributed to this thesis, both directly and indirectly. During my undergraduate experience at Oakwood University, I was tremendously grateful for the exquisite mentorship of Dr. Safawo Gullo, who encouraged me to seek out opportunities that would enhance my scientific career and provided support for all my professional endeavors. As an undergraduate, I had the unique opportunity to work with Dr. Samer Hattar at Johns Hopkins University for two summers. This opportunity was made possible through the SURE (summer undergraduate research experience) led by Dr. Randriamahefa of

Oakwood University. With the stellar mentorship and support I received from Dr. Hattar over the summers of 2007 and 2008, I gained the experience and knowledge I needed to apply and gain acceptance into the CMDB program in Biology department of Johns Hopkins University. Dr. Hattar, who became my graduate school thesis advisor, believed in me, guided me and gave me the tools and support I needed to succeed.

Although graduate school has been arduous, to say the least, I have had an amazing experience working with the members of the mouse tri-lab. Professors, post-docs, and students alike, never hesitated to lend a hand, an ear, or wise council whenever I needed. I want to thank Dr. Diego Fernandez for being one of my primary sources for listening to my countless scientific brainstorming conversations. I have made some wonderful life-long friends while in the tri-lab: Chantal Bodkin Clarke, Ami Patel (Shah) and Chih-Ming Chen. These were definitely the people who provided the daily emotional support and laughs I needed to survive on the cloudy days.

Outside of the lab, I had a very large and loving church family that adopted me as their sister, friend, and daughter. Last but not least, I want to thank the love of my life, my fiancé, Frederick Richardson. Frederick was not only emotionally supportive during this process, but also revised my presentations and gave me scientific feedback. I can

honestly say that Frederick has been a very positive force in the completion of my thesis.

Finally, I want thank and dedicate to my thesis to Marishka, my sister, April, my mother, and Emmanuel Simmonds, my father. I truly believe I have the most supportive family in the world. For as long as I can remember, my parents have always given me the freedom of thought, love, and their last dime, to support all of my academic endeavors. Whether or not they knew exactly what I was doing, they always made me feel like a superstar, which gave me the courage and drive to keep moving forward. I also thank Michael Hinds, a close friend of my family, who has played a major supportive role throughout my college and graduate school career. There was not a moment that I thought any of my goals were impossible. The support of family and friends is a power that should never be underestimated.

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LIST OF ABBREVIATIONS

SCN- Suprachaismatic Nucleus

ipRGCs- intrinsically photosensitive Retinal Ganglion Cells

OPN- Olivary pretectal nucleus

VLPO- Ventral lateral preoptic area

RGCs- Retinal ganglion cells

RT-PCR- Reverse transcription-polymerase chain reaction

DTA- Diptheria toxin A subunit

POU- Pit-Oct-Unc

PLR- Pupillary light reflex

IGL- Intergeniculate leaflet

LGv- Lateral geniculate nucleus

LGN- Lateral geniculate nucleus

LD – light-dark

SAD- Seasonal affective disorder

LL- Constant light

DD- Constant darkness

Frq- *Frequency*

TIM- *Timeless*

PFA- Paraformaldehyde

AVP- Arginine-vasopressin

VIP- Vasoactive intestinal peptide

MKO- Melanopsin knockout

WT- Wild type

RKO- Rod knockout

ZT- Zeitgeber time

CT- Circadian time

Chapter 1: INTRODUCTION

Circadian rhythms in a modern society

Circadian rhythms are endogenous rhythms on a daily cycle of approximately 24 hours. In order for organisms to establish and maintain temporal synchrony with their environment, they must precisely align their circadian rhythms of about 24 hours to the external light dark cycle of exactly 24 hours (Turek *et al.*, 2005). This light-driven alignment of circadian rhythms to the light-dark environment is known as circadian photoentrainment. In mammals, circadian photoentrainment is primarily mediated by light input from the retina to the master biological clock, the suprachiasmatic nucleus (SCN). The SCN is a cell body rich, heterogeneous nucleus located in the mammalian ventral hypothalamus. In addition to the SCN, numerous peripheral biological clocks also regulate physiological homeostasis. These peripheral clocks are synchronized by the SCN (Reviewed in Buijs and Kalsbeek 2001). Without this synchronization, peripheral biological clocks could not work together in the timely manner necessary to carry out a range of bodily functions.

Circadian photoentrainment can be socially appreciated by the complementary sleep-wake cycles of most people, which allow a civilized society to facilitate trade, education, and social interaction at specific times of the day (i.e. day vs night). Within the past century, there has been a vast expansion of new technologies that interfere with the natural

light-dark environment. Inventions such as artificial lighting, airplanes, and electronics are important components in the success of our society. However, exposure to artificial light at night and rapid air travel across multiple time zones disrupt the natural light-dark environment, which leads to major impacts on the health of individuals across the globe (Schernhammer *et al.,* 2001; Schernhammer *et al.,* 2003; Filipski *et al* 2004). For example, light exposure at night can disrupt sleep, which leads to irritability and poor focus in daily activities. Additionally, light exposure at night often results from shift working, where many people are awake and at work instead of sleeping at night. Therefore, maintenance of a photoentrained circadian rhythm in modern society is challenging. The solution for this dilemma is to either return to a time without our current technologies, or understand the circadian system to a level that we can manipulate the system to adapt to our changing environment. The latter is my aim.

Day length and circadian rhythms

Changes in day length occur over the course of a year, although the natural light-dark cycle is largely consistent from day to day. Most variation in day length occurs closer to the north and south poles. Thus, the extent of the seasonal change in day length is dependent on latitude. Many studies have attempted to assess the effects of the seasonal changes in day length on social behavior in animals and on emotional

distress in humans (Dittami 1981; Golder and Macy 2011). Some studies have also investigated the physiological changes in the SCN in relation to season changes (Reviewed in Coomans *et al.,* 2014). There is not much known about how light from the external environment regulates these changes in the SCN. Although circadian photoentrainment refers to the daily alignment of circadian rhythms, it is less understood whether the body similarly regulates circadian photoentrainment in different day lengths. For instance, does one recover from jet lag during the winter months the same as they would during the summer months? My thesis aims to answer these questions.

Historical studies on the effects of aberrant light on circadian photoentrainment

In 1958, a study reported that light pulses of different intensities administered at specific times of the circadian cycle can explain photoentrainment of the luminescence rhythm in the dinoflagellate, *Gonyaulax polyedra* (Hastings and Sweeny 1958). Depending on the time of day at which the light pulse is presented, an earlier or later shift in the phase of the circadian rhythm may occur. These pioneering studies led to the idea that circadian photoentrainment occurs due to the summation of sudden daily shifts in the phase of the circadian clock (Daan 1977, DeCoursey 1986, Johnson 1999, and Yamanaka *et al* 2014), in what is referred to as the non-parametric model of circadian photoentrainment

(Pittendrigh and Daan 1976a). In contrast, the parametric model of circadian photoentrainment proposes that once an endogenous threshold at which the clock can respond to light is reached, prolonged light exposure can lengthen or shorten the period of the clock, resulting in entrainment to the 24-hr external environment (Aschoff *et al* 1971).

In addition to phase shifting, light may directly regulate behavior to aid in photoentrainment. One such effect is known as "negative masking" (Mrosovsky 1999), which refers to the suppressive effect of light on behavior in nocturnal animals and is thought to be independent of circadian control. It is still unknown whether and how the direct effects of light and the phase shifting effects of light interact to drive circadian photoentrainment.

Light reception of the retina: Rods, Cones, and ipRGCs

In mammals, light input from the retina entrains the circadian pacemaker located in the SCN. The retina is known to contain three types of photoreceptors: the classical photoreceptors, rods and cones, and the intrinsically photosensitive retinal ganglion cells (ipRGCs), a unique population of photosensitive retinal ganglion cells (RGCs) (Hattar *et al* 2003). ipRGCs are photosensitive due to the expression melanopsin. ipRGCs are necessary for circadian photoentrainment, as normal circadian photoentrainment is abolished in their absence (Guler *et al* 2008; Goz *et al* 2008; Hatori *et al* 2008). As ganglion cells, ipRGCs also

receive rod and cone input (Wong 2012; Schmidt *et al* 2011). Therefore, ipRGCs are responsible for driving circadian photoentrainment due to the intrinsic photosensitivity of ipRGCs and extrinsic input from rods and cones (Altimus *et al.,* 2010; Lall *et al.,* 2010). Although melanopsin is the defining protein of photosensitivity in ipRGCs, most studies have reported that melanopsin has a minimal role in circadian photoentrainment.

Discovery and characterization of melanopsin

Melanopsin is a G-protien coupled receptor that was first discovered and isolated from the light sensitive dermal melanophores in *Xenopus* (Provencio et al., 1998). Soon after its discovery, melanopsin was shown to act as a photopigment in mammalian retina (Berson and colleagues in 2002). They demonstrated that a subset of RGCs is capable of responding to light with no rod or cone input and hence are intrinsically photosensitive (now known as ipRGCs). These ipRGCs were later found to express the photopigment melanopsin, which renders them photoreceptive (Berson et al., 2002; Hattar et al., 2002). The identification of ipRGCs in the mammalian retina reconciled earlier reports that mice genetically modified to lack functional rods and cones can photoentrain and constrict their pupils to light (Lall et al. 2010; Freedman et al., 1999). Therefore, in addition to the previously discovered photoreceptor classes, ipRGCs define a third class of photoreceptors in the mammalian retina (Berson et al., 2002; Hattar et

al., 2002). The spectral sensitivity of melanopsin has been an area of conflicting views in the melanopsin field (Newman et al., 2003; Melyan et al., 2005; Panda et al., 2005; Qiu et al., 2005, Yoshimura and Ebihara, 1996, Provencio et al., 1998). Animals that completely lack rods and cones (rd/rd; cl), and hence depend only on ipRGCs for light detection, showed an action maxima of 480nm, which is currently accepted spectral maxima for melanopsin (Hattar et al., 2003; Lucas et al., 2003; Panda et al., 2005; Qiu et al., 2005).

Circuits for light reception: ipRGC subtypes

At least five distinct subtypes of ipRGC subtypes have been determined based on dendritic morphology, stratification, soma size, and electrophysiological properties (Schmidt *et al.,* 2011). The ipRGCs subtypes, M1-M5, were characterized in the mouse retina where they comprise only 5% of the total RGC population (Ecker *et al.,* 2010). The M1 ipRGCs is most characterized subtype. M1 ipRGCs primarily target the SCN, OPN and IGL while M2-M5 ipRGCs innervate areas necessary for image formation (Ecker *et al.,* 2010). Studies from the Hattar lab using an attenuated diphtheria toxin A subunit (aDTA) to ablate ipRGCs indicate that the SCN is innervated predominantly by the M1 subtype. Furthermore, even M1 ipRGCs are divided into molecularly distinct subpopulations based on the expression of the POU domain transcription factor, Brn3b (Chen *et al.,* 2011), which is involved in RGC differentiation

(Badea *et al.,* 2009). The majority of M1 express Brn3b, whereas only 200 M1 ipRGCs do not express Brn3b. The SCN is predominantly innervated by the 200 M1 ipRGCs that lack Brn3b, whereas the IGL and the OPN receive input predominantly from Brn3b-positive ipRGCs (Chen *et al.,* 2011). These 200 Brn3b-negative M1 ipRGCs are capable of driving circadian photoentrainment at high light intensities (Chen *et al.*, 2011).

Light reception by the brain: SCN versus non-SCN regions

The SCN is the only known ipRGC target that relies exclusively on the retinal light input mediated by ipRGCs; in their absence, all light driven functions are abolished. In contrast, the pupillary light reflex is severely attenuated, but not completely abolished in the absence of ipRGC input to the OPN. The IGL has been implicated in circadian photoentrainment and masking in work by Nicholas Mrosovsky (Mrosovsky *et al* 1999). In addition to the SCN, IGL, and OPN, ipRGCs project to brain regions known for their involvement in visual-related functions (ventral division of the lateral geniculate nucleus (LGv), superior colliculus, preoptic area, peri-supraoptic nucleus, and posterior limitans nucleus), the limbic system (medial amygdala) and behavioral regulation (lateral nucleus and subparaventricular zone of the hypothalamus, margin of the lateral habenula, and periaqueductal gray) (Hattar *et al.,* 2006). The role of light in driving the function of most of these ipRGC targets remains to be determined. The study of the

functional importance of the ipRGC mediated light input to each ipRGC target is limited by the lack of tools to identify and isolate each ipRGC subtype *in vivo*. Thus far, using the Brn3bzDTA/opn4Cre line, work from the Hattar lab has reported that the OPN receives input exclusively from the Brn3b-positive ipRGCs (Chen *et al.,* 2011). Using the opn4LacZ line, these ipRGCs were identified as the M1 subtype. Future studies are necessary to determine the contribution of each ipRGC subtype to what seems (from the innervation pattern) like a broad range of light-regulated behaviors. In chapter 4, I discuss my findings on the role of melanopsin in the Brn3b-negative M1 ipRGCs in regulating light driven circadian responses.

Light effects on circadian related functions

Light input to the brain is not only important for circadian photoentrainment, but also for modulating behaviors in response to acute and chronic changes in the light-dark (LD) environment. One of the most interesting aspects of light influencing human behavior is that of the shorter day length that occurs in the winter months. As a consequence of the short day, some humans develop a seasonal form of depression known as seasonal affective disorder (SAD), which can be ameliorated by exposing affected individuals to bright light (Pail *et al.*, 2011). An exciting study using human subjects has implicated a mutation in the melanopsin gene with higher incidence of SAD (Roecklein

et al., 2009), providing the intriguing hypothesis that lack of melanopsin signaling could lead to SAD. Recent work from the Hattar lab demonstrated that ipRGCs play a crucial role in the depressive effects of aberrant light cycles (LeGates *et al.,* 2012). My studies could provide mechanistic insights on how short days lead to SAD in humans.

In addition to chronic light effects on human behavior, it is also well known that acute light exposure at night in humans causes strong suppression of melatonin in a light intensity dependent manner (Lewy *et al.,* 1985). This suppression of melatonin reduces the drive for sleep causing sleep deprivation and sleep fragmentation in humans exposed to bright light at night (Gooley *et al.,* 2011). Understanding how animals respond to acute light treatments will be relevant for determining the circuits underlying how bright light at night causes sleep problems in humans. An established behavior that mimics melatonin suppression in humans is activity reduction in mice that are exposed to light at night (Mrosovsky and Hattar 2003). Mice are active at night and short pulses of acute light treatment reduce mice activity directly in what is known as masking (since light *masks* the drive of the circadian clock for mice to be active at night; Mrosovsky 1999). I will utilize light pulses at night to determine the contribution of Brn3b-negative and Brn3b-positive ipRGCs to the acute effects of light on behavior. This behavior is quite interesting, since for shorter pulses of light, the melanopsin protein is dispensable, whereas for longer durations (similar to LL), the melanopsin

protein is required (Mrosovsky and Hattar 2003). This will allow me, using the same behavior, to determine if distinct subpopulations of ipRGCs contribute to this function at different durations of light stimulations. Combined, my studies will allow me to delineate the functional importance of distinct ipRGC subtypes and the rod/cone input to a wide array of clinically important light-dependent physiological functions.

The clinical significance of understanding the light pathways that mediate non-image forming behaviors

Concurrent with new scientific discoveries is the production and administration of treatments for an array of illnesses. While most of these medical advances have saved many lives, they come with side effects and do not work for everyone. Although there are moderate variations between individuals that may account for how well a treatment works, there is still much unknown about the basic mechanisms underlying key organism functions. Therefore, while we celebrate advances in treatment options, even more research must be conducted to decipher both the internal biological pathways as well as how the external environment affects homeostasis. Therefore, this current body of work takes a closer look at how the rods, cones and melanopsin relay light information through a specific population of ipRGCs to a specific brain target.

Aberrant light exposure can result in array of symptoms such as: depression, insomnia, anxiety, irritability, learning and memory deficits as well as an array of circadian dysfunctions. Since ipRGCs project to a wide array of brain regions, it is difficult to know which regions are playing a role in mediating the light information that results in the symptoms caused by aberrant light exposure. Additionally, ipRGCs are a heterogeneous population of cells and therefore most likely transmit different kinds of light information to the brain. Therefore, it is crucial to develop a system where we isolate a single ipRGC subtype that targets a single ipRGC target. This will allow us to systematically study the role of light input to different regions of the brain. In my dissertation, I addressed this issue by using a mouse model in which only one ipRGC-subtype was present that exclusively projected to the SCN. I was able to study the contribution of the blue wavelength activated melanopsin photopigment *in vivo*, through the SCN-projecting M1 ipRGCs. Additionally, I was able to understand more about how this circuit is involved in relaying information about changes in the light-dark environment. We found an important role for melanopsin in circadian functions that were previously considered as well-defined. My work opens the door for even more targeted treatments for light induced circadian related dysfunctions.

Chapter 2: The contribution of melanopsin to the circadian photo-responsiveness of SCN cells

INTRODUTION

The phase of circadian rhythms can be shifted by light presented at specific times of the day. In nocturnal animals like mice, light exposure during the early night delays the clock, while light exposure during the late night advances the clock. Phase advances and phase delays are of a similar magnitude in invertebrate systems, which may suggest that light may regulate these responses in a similar manner (Crosthwaite *et al.,* 1995; Suri *et al.,* 1998). However, in both diurnal and nocturnal mammals, brief exposure to light induce phase advances and phase delays of different magnitudes (Daan 2000). For example, in nocturnal animals such as hamsters, maximal phase advances tend to be much smaller (~30-60 mins) while maximal phase delays are much larger (~120 mins or more) (Mistlberger and Antle 2011). There is not much known of how light stimulates SCN clock cells to drive phase advance and phase delays. Therefore, my goal was to determine whether the difference in phase shift direction and magnitude was due to differential activation during the early night versus late night of immediate early genes such as c-fos and pS6, which is known to reflect the light-driven activation of SCN cells. Protein expression of both c-fos and pS6 peak around 90 minutes following light exposure (Rea 1989; Schwartz *et al.,* 1996; Knight *et al.,* 2012). In the dark, there is no activation of c-fos or pS6 in the SCN. Additionally, previous studies have established regional areas of the SCN based on the differential expression

of neuropeptides such as arginine-vasopressin (AVP) and vasoactive intestinal peptide (VIP) (Abrahamson and Moore 2001). It remains to be determined whether the light driven activation of c-fos and pS6 is localized to specific SCN cell populations like AVP and VIP.

Phase shifting of circadian rhythms occurs when external light information mediated by the rods, cones and ipRGCs. Rods and cones provide extrinsic light input through ipRGCs to the SCN while the photopigment melanopsin provides the intrinsic light responses of ipRGCs to the SCN. It has been determined that the presence of melanopsin in ipRGCs is important for phase shifting in response to early night light exposure (Panda *et al.,* 2002). While melanopsin may be important for phase delaying the clock, not much is known about the influence of the retina on phase advancing. Therefore, I predict that light differentially activates the SCN during the early night as compared to the late night and that melanopsin may play an important role. By studying the contribution of melanopsin in phase shifting, we can hope to eventually develop methods to induce or avoid phase shifting in our own environment.

METHODS

To test my prediction, I investigated the induction of the immediate early gene, c-fos, in response to light during the early night versus late night. During the experiment mice were kept in a 12 hr light: 12 hr dark cycle (12:12 LD). 15 mins of light was administered at either 2 hours after dark onset (ZT-zeitgeber time 14) or 10 hours after dark onset (ZT 22). Following light exposure, mice were returned to the darkness for 90 mins, after which they were anesthetized and perfused through the heart with 4% PFA in PBS. Isolated brains were post-fixed overnight in 4% PFA at 4 C. 50um sections of the SCN were collected using the vibratome for the c-fos staining. 25um sections of the SCN were collected using the cryostat for the pS6 staining.

Immunohistochemistry (IHC) for c-fos

Coronal brain sections (30-40 um) were distributed into wells containing PB (2-4 sections per well). Sections were incubated overnight in 400 uL of rabbit anti-cFos, from Calbiochem Ab-5 (1: 15, 000 uL in blocking buffer (BB)- 0.1M PB + 3% Triton X-100 + 0.5% BSA + serum from Vectastain kit (3 drops/10 mL or 150 uL/10mL). Sections were then washed 3 times with 500 uL of rinse buffer (RB)- (0.1M PB + 3% Triton X-100) (5 minutes per wash). The last wash was replaced with 400 uL goat anti-rabbit (from vectastain kit) (1 drop in 10 mL BB), and incubated for 1 hour, rocking at room temperature. The sections were

then washed 3 times with 500 uL RB (5 minutes per wash). The last wash was replaced with 400 uL ABC reagent and incubated for 1 hour, rocking at room temperature. Sections were washed 3 times in 500 uL of RB (5 times) before being developed with DAB (10 mg of DAB powder per 20 mL of solution Tris-buffered saline pH 7.6 and 16uL 30% hydrogen peroxide).

Immunuhistochemistry for pS6, VIP and AVP

Co-stained pS6 with antibodies against arginine-vasopressin (AVP) or vasoactive intestinal peptide (VIP) using the following protocol to determine whether the light activated cells were region specific. Tissue were fixed at room temperature for 2 hrs in 4% PFA in PBS, blocked in 7% donkey serum in 0.3% triton in PBS and incubated in the primary antibody overnight at 4-degrees Celsius. The secondary antibody used was either donkey α rabbit-546 or 488 at a dilution of 1:200. Primary antibody dilutions: rabbit α PS6 at 1:300, rabbit α AVP at 1:800, and rabbit α VIP at 1:500.

Cell counts

c-fos positive SCN cells were labeled with one dot per cell using a PowerPoint, after which a binary file of the dot file for each 50um section of the SCN was created using ImageJ64. Cells counts were calculated from the binary file using ImageJ64. All sections of the SCN for each

animal in each experimental group were aligned rostrally to caudally before graphing in Prism Graphpad.

The fluorescent secondary used in the pS6, AVP and VIP protocols, required a different approach to quantification as compared to the how c-fos positive SCN cells were quantified. Cell counts for fluorescence were done manual and visually 1-2 times each by both Kristoff Foster (my summer undergraduate student) and myself. Each section for the immunofluorescence staining was 25um, half the size of that for c-fos IHC, therefore, we pooled the cell counts for the rostro-caudal analysis of the SCN by averaging every two sections of the SCN. The final result for each of the 10 points on the graph is approximately half of the actual cell count per 50um. We decided against adding the cell counts from each of the 25um sections because some of the SCN sections were lost during the sectioning process on the cryostat. The fluorescent data was also graphed using Prism Graphpad.

RESULTS

Light exposure during the early night activates c-fos in fewer cells in the SCN in a melanopsin dependent manner.

In the SCN of WT animals, c-fos expression is observed more in the mid and caudal SCN during the early night (ZT 14) while there is expression along the entire rostro-caudal SCN axis during the late night (ZT 22) (Figure 1A, 1C and 1D). No cells are immunostained for c-fos in the SCN in the absence of a light stimulus at night (Figure 1B). In the melanopsin knockout (MKO) animals, where the melanopsin protein is absent in all ipRGCs, and thus rods and cones provide the only light input through ipRGCs, there are significant differences in the number of c-fos postive cell in the SCN during the early night versus late night in response to light (Figure 2A). Fewer cells are immunostained at early night than late night in MKO animals (Figure 2B). The most significant reduction in c-fos positive cells is observed in the rostral and mid-caudal (approximately 2/3 of the distance through the SCN heading rostrally to caudally) region of the SCN (Figure 2C).

To determine whether melanopsin was playing a role in light response in the SCN, I compared the number of c-fos positive cells in the SCN of MKO and WT animals during the early night and late night. MKO animals have fewer c-fos positive SCN cells in response to light during the early night in the mid-caudal region of the SCN (Figure 3A-B). In

contrast, there is a similar number of c-fos positive SCN cells in response to light exposure during the late night MKO and WT animals (Figure 3C-D). In addition to the differences observed in the c-fos expression along the rostro-caudal axis in both the WT and MKO animals, there is also a dorso-ventral difference in the expression pattern of c-fos in response to light. These differences are observed in the mid-caudal SCN between the early night and late night, in both the WT and MKO animals, where there are more c-fos positive cells in the dorsal region of the SCN during the late night as compared to the early night (Figure 3A and 3C).

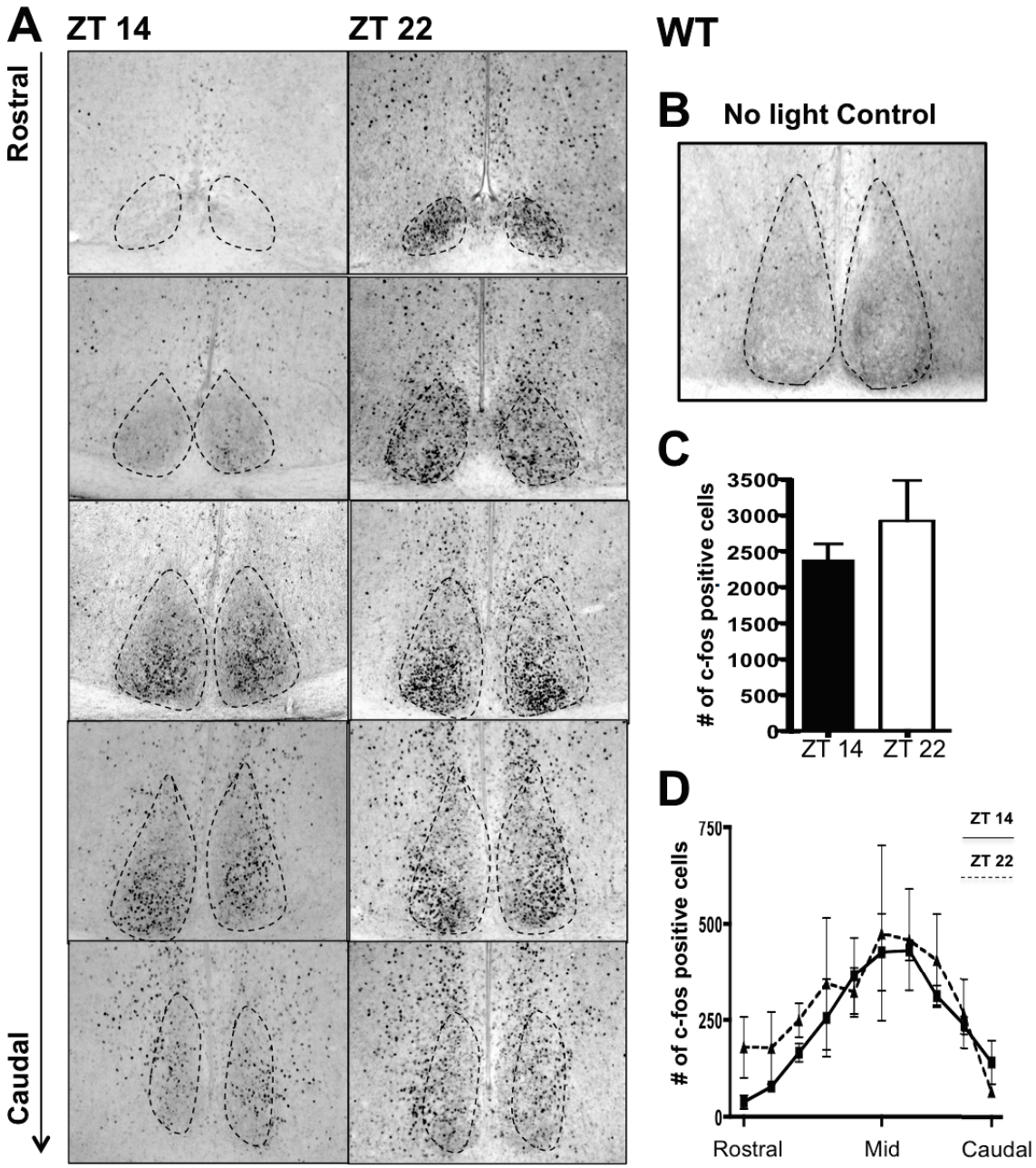


FIGURE 1: There is a moderate difference in the amount of c-fos produced by light during the early vs late night in WT animals in the rostral SCN.

A) c-fos induction by 15 mins of light in SCN of WT animals at ZT14 and ZT 22. 50 um rostral to caudal sections of the SCN. Every second section shown. B) No c-fos expression is observed in the absence of a light stimulus. C) Quantification of total c-fos positive cells at ZT 14 and ZT 22 in SCN of WT animals. Distribution of c-fos positive cells in a series of coronal sections across the rostro-caudal extent of the SCN at ZT 14 and ZT 22 in WT animals.

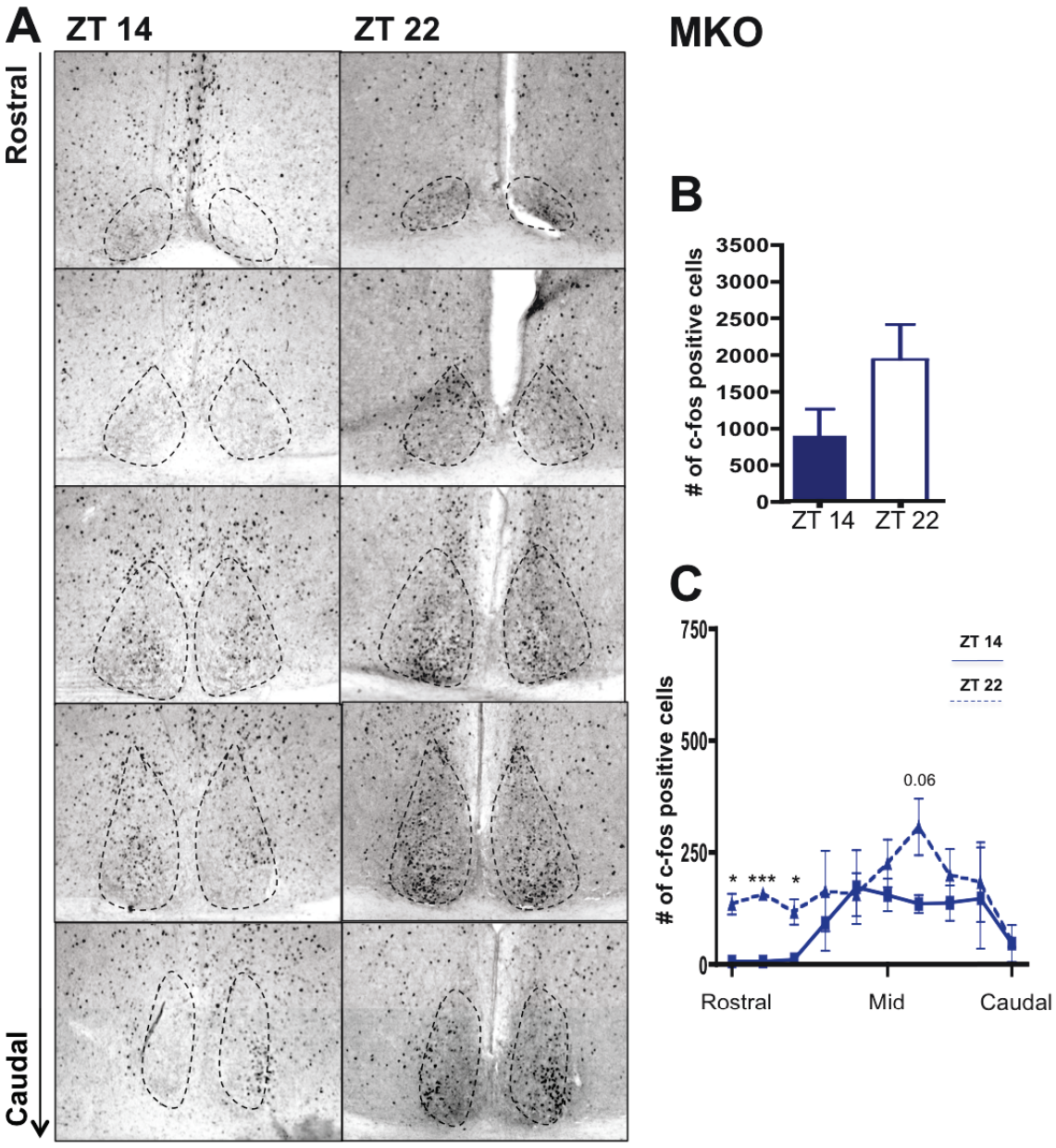


FIGURE 2: C-fos induction by light during the early vs late night varies in specific regions of MKO SCN.

A) c-fos induction by 15 mins of light in the SCN at ZT14 and ZT 22 of MKO animals. 50 um rostral to caudal sections of the SCN. Every second section shown. B) Quantification of total c-fos positive cells at ZT 14 and ZT 22 in the SCN of MKO animals. C) Distribution of c-fos positive cells in a series of coronal sections across the rostro-caudal extent of the SCN at ZT 14 and ZT 22 in MKO animals. Statistical analyses: Two-way ANOVA. P-values: *P<0.05, ***P<0.001.

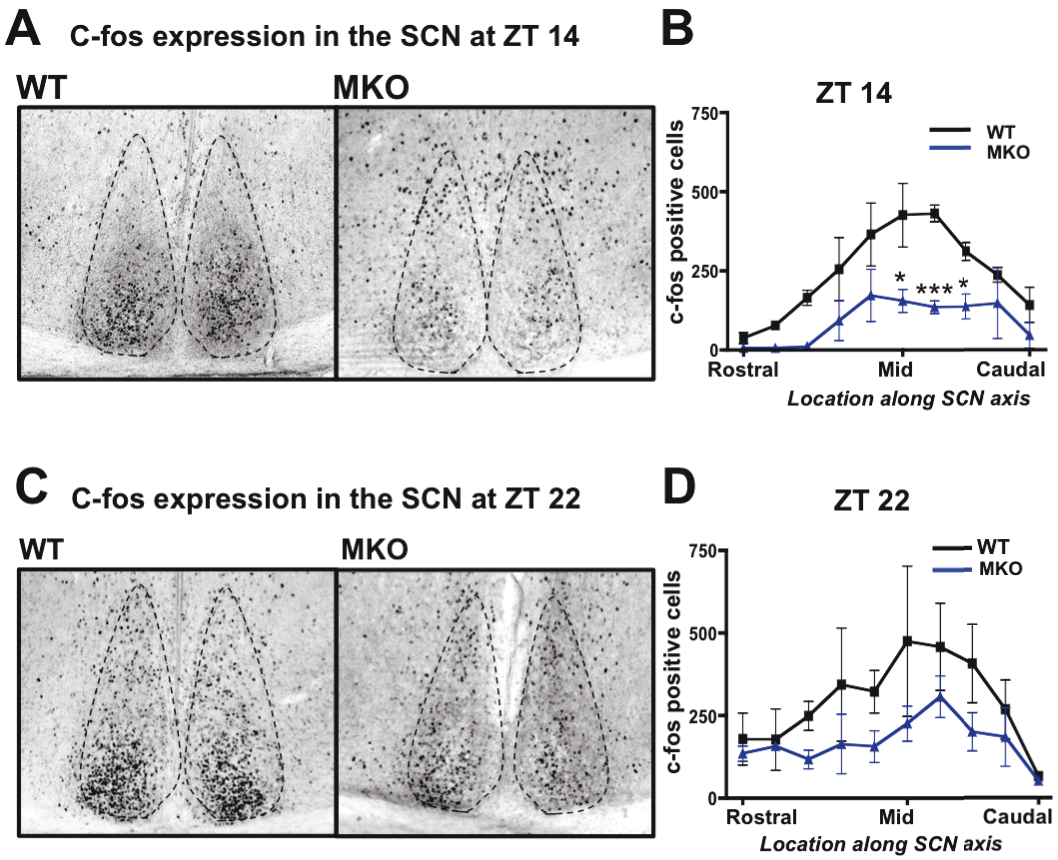


FIGURE 3: C-fos induction by light is less in MKO animals during the early but not late night.

A) c-fos induction by 15 mins of light in the SCN of WT and MKO animals at ZT14. 50 um mid-SCN section shown. B) Rostral to caudal distribution of c-fos positive cells at ZT 14 in the SCN of WT and MKO animals. C) c-fos induction by 15 mins of light in the SCN of WT and MKO animals at ZT 22. 50 um mid-SCN section shown. D) Distribution of c-fos positive cells in the mid-caudal extent of the SCN at ZT 22 in WT and MKO animals. Statistical analyses: Two-way ANOVA. P-values: *P<0.05, ***P<0.001.

Melanopsin influences the expression of pS6 in the SCN in a temporal and cell-type specific manner.

The rostro-caudal, dorso-ventral and melanopsin dependency of c-fos expression in the response to light during the early night versus late night, led to the thought that different populations of SCN cells may respond to light at night. AVP and VIP are two peptides that are expressed in the ventral and dorsal region of the SCN, respectively. Cells that express AVP or VIP has been thought to play an important role in SCN light reception and transmission to other brain regions (Romijn *et al.,* 1997; Maywood *et al.,* 2006). In order to determine the expression pattern of c-fos in AVP and VIP cells, a double immunofluorescence approach was necessary.

While our c-fos antibody worked well for immunofluorescence in other brain regions, staining in the SCN is very weak. Therefore, we used an antibody against the phosphorylated form of the ribosomal protein S6, which is induced by light in the SCN in a circadian specific manner (Knight *et al.,* 2012). In contrast to the similar number of c-fos positive cells in the SCN in WT animals during both the early night and late night, there are significantly more pS6 positive SCN cells during the late night as compared to the early night in WT animals (Figure 4A, C-D). There is no expression of pS6 in the SCN in the absence of a light stimulus (Figure 4B). In contrast to the difference in c-fos expression in

MKO animals between the early night versus late night, there is no difference in the number of pS6 positive cells in the SCN during the early night and late night in MKO animals (Figure 5).

To determine whether melanopsin expression in ipRGCs was important for pS6 induction in the SCN, we analyzed pS6 expression in MKO animals during the early night and late night (this work was done in collaboration with my summer student Kristoff Foster). In contrast to the fewer c-fos positive cells we observed in the SCN on MKO animals as compared to WT animals, there is no difference in the number of SCN cells that expressed pS6 in response to light during the early night between WT and MKO animals (Figure 6A-B). There is an increase in the number of pS6 positive cells in WT animals as compared to MKO animals at during the late night (Figure 6B-C). These data suggests that while both c-fos and pS6 are activated by light, their expression pattern in response to light is different. Melanopsin is important for c-fos expression during the early night as well as pS6 expression during the late night.

To determine whether the pS6 expression was unique to either AVP or VIP expressing SCN cell types, pS6 was co-stained with either AVP or VIP. Compared to WT animals, there are more AVP and VIP cells in the MKO animals that are activated by light (pS6 positive) during the early night (Figures 7 and 8). While there were dramatic differences in

pS6 induction between MKO and WT animals during the late night, we found no differences in the number of AVP and VIP cells that were also pS6 positive (Figures 9 and 10). These data suggest the cells activated by light in a pS6-dependent manner generally express neither AVP nor VIP. It is however possible that the c-fos positive cells could be of the AVP or VIP cell population.

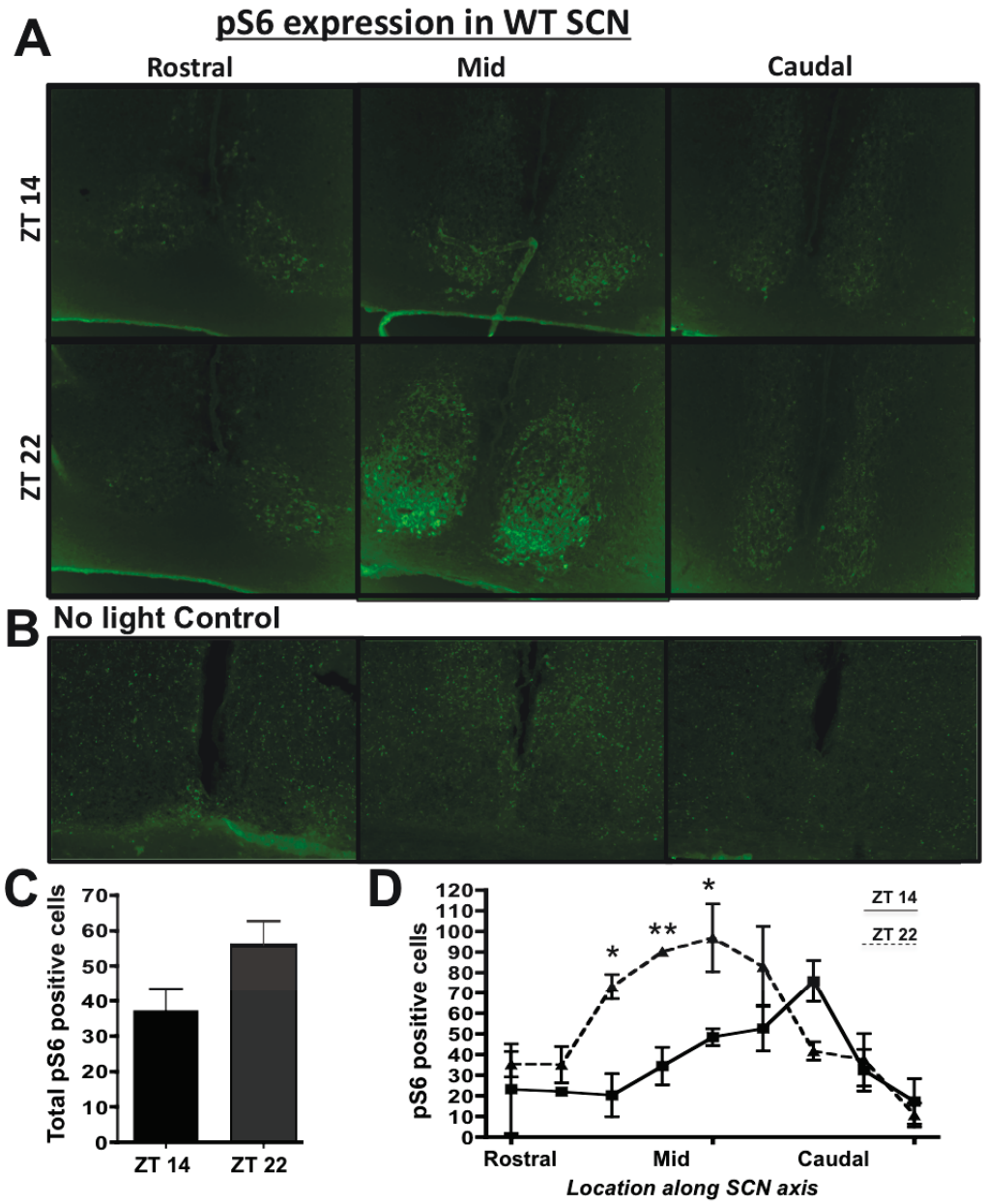


FIGURE 4: pS6 induction by light is up-regulated in the mid-SCN during the late night in WT animals.

A) pS6 induction by 15 mins of light in the SCN of WT animals at ZT14 and ZT 22. 25 um rostral, mid, and caudal SCN sections shown. B) No light pulse control. C) Quantification of total pS6 positive cells at ZT 14 and ZT 22 in WT SCN. D) Rostral to caudal distribution of pS6 positive cells at ZT 14 and ZT 22 in WT SCN. Statistical analyses: Two-way ANOVA. P-values: *P<0.05, **P<0.01.

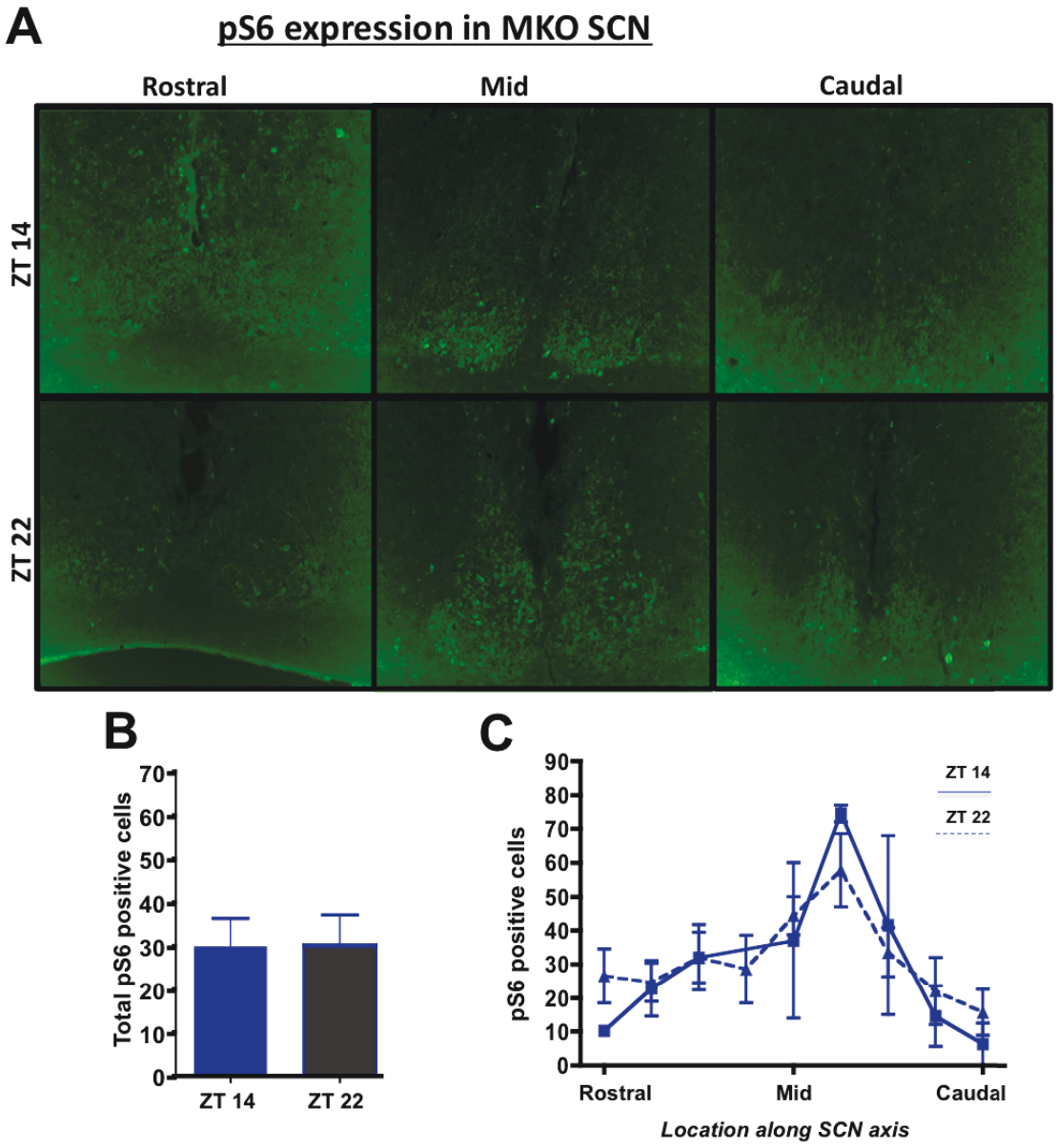


FIGURE 5: There is no difference in the pS6 induction by light the MKO SCN during the early or late night.

A) pS6 induction by 15 mins of light in the SCN of MKO animals at ZT14 and ZT 22. 25 um rostral, mid, and caudal SCN sections shown. B) Quantification of total pS6 positive cells at ZT 14 and ZT 22 in the SCN of MKO animals. C) Rostral to caudal distribution of pS6 positive cells at ZT 14 and ZT 22 in the SCN of MKO animals.

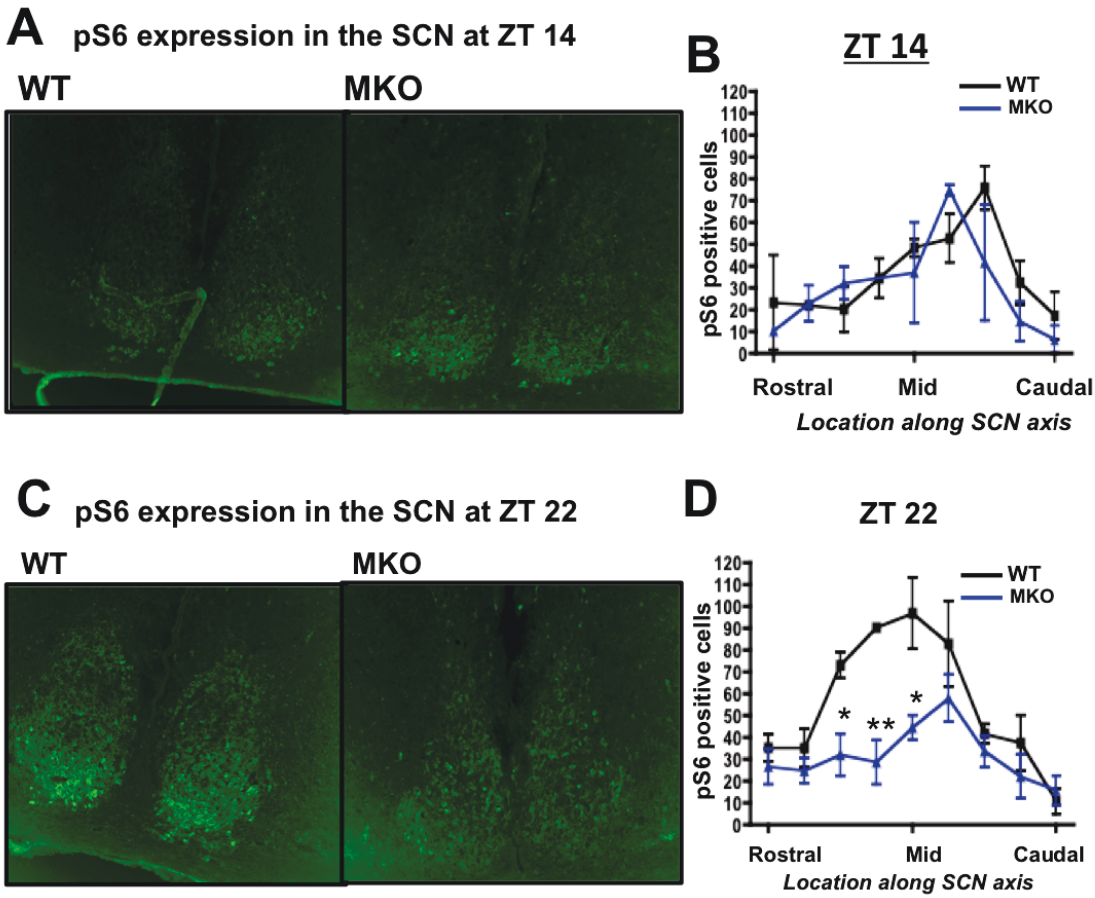


FIGURE 6: pS6 induction by light is less in MKO animals during the late but not early night.

A) pS6 induction by 15 mins of light in the SCN of WT and MKO animals at ZT14. 25 um mid-SCN shown. B) Rostral to caudal distribution of pS6 positive cells at ZT 14 in the SCN of WT and MKO animals. C) pS6 induction by 15 mins of light in the SCN of WT and MKO animals at ZT 22. 25 um mid-SCN section shown. D) Rostral to caudal distribution of pS6 positive cells at ZT 22 in the SCN WT and MKO animals. Statistical analyses: Two-way ANOVA. P-values: *P<0.05, **P<0.01.

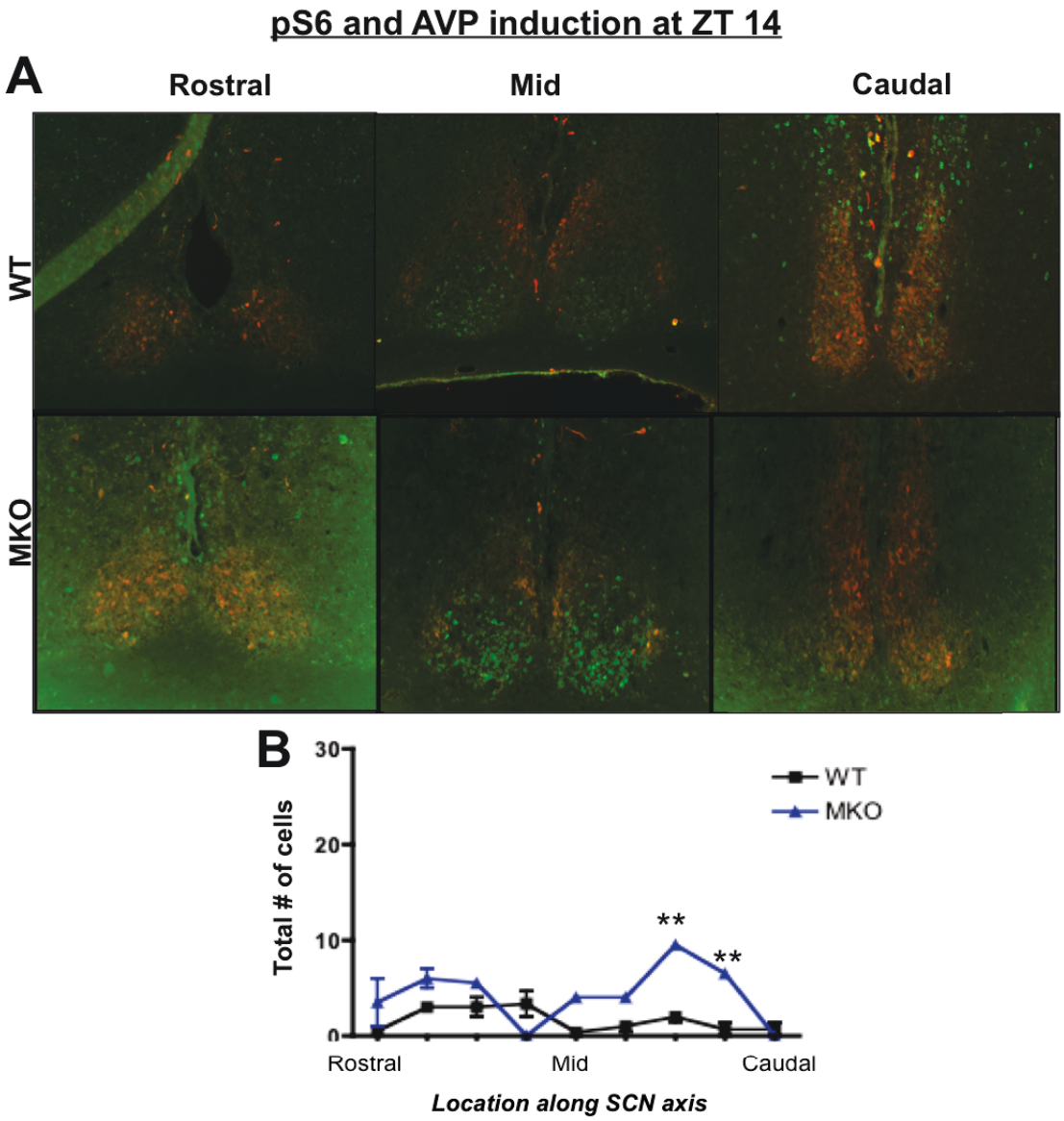


FIGURE 7: MKO animals have more AVP cells that are pS6 positive than WT animals during the early night.

A) AVP co-stained with pS6 following 15 mins of light in the SCN of WT and MKO animals at ZT14. 25 um SCN sections shown. B) Rostral to caudal distribution of co localized AVP and pS6 positive cells at ZT 14 in the SCN of WT and MKO animals. Statistical analyses: Two-way ANOVA. P-values: **P<0.01.

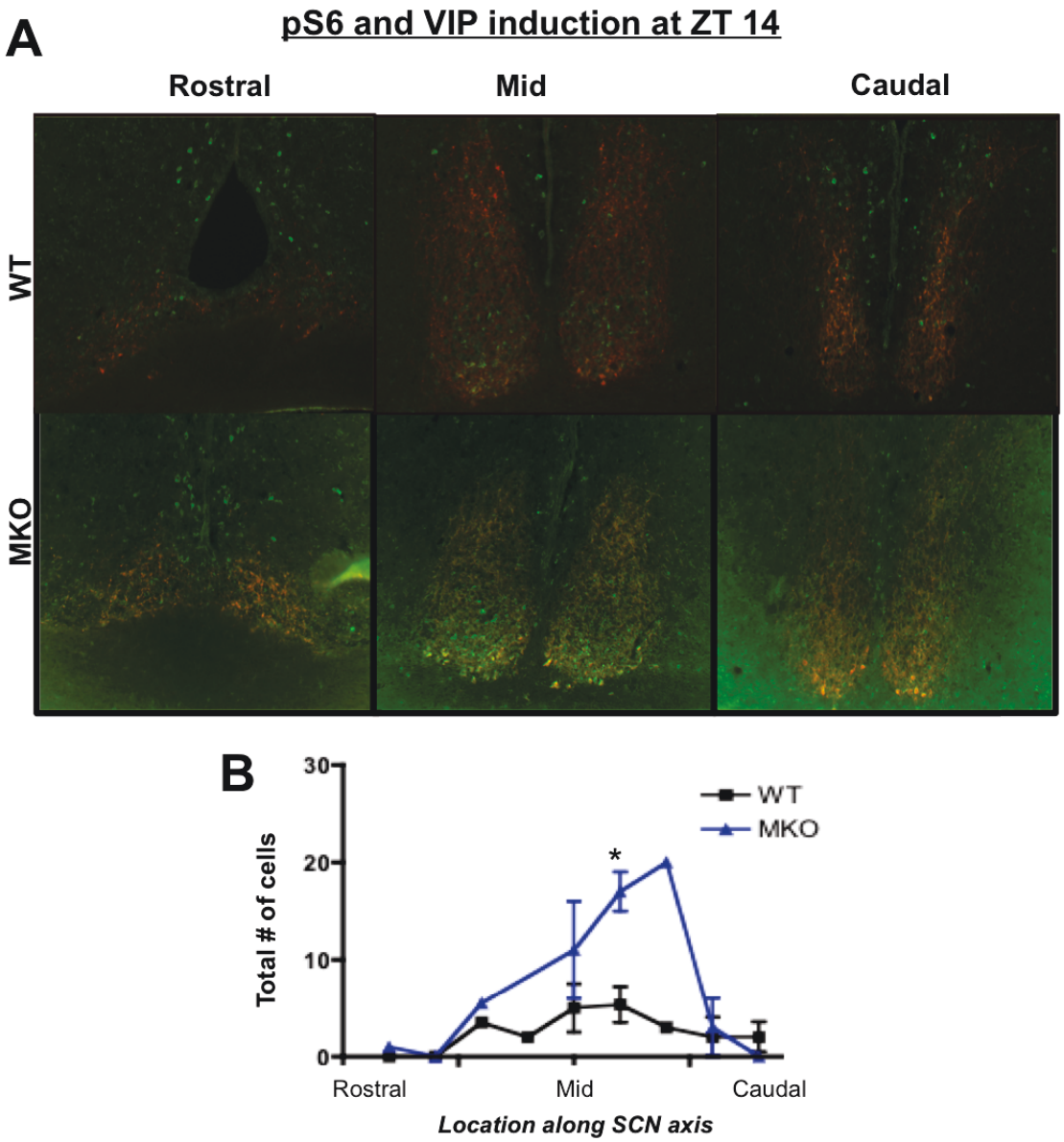


FIGURE 8: MKO animals have more VIP cells that are pS6 positive than WT animals during the early night.
A) VIP co-stained with pS6 following 15 mins of light in the SCN of WT and MKO animals at ZT14. 25 um SCN sections shown. B) Rostral to caudal distribution of co localized VIP and pS6 positive cells at ZT 14 in the SCN of WT and MKO animals. Statistical analyses: Two-way ANOVA. P-value: *P<0.05.

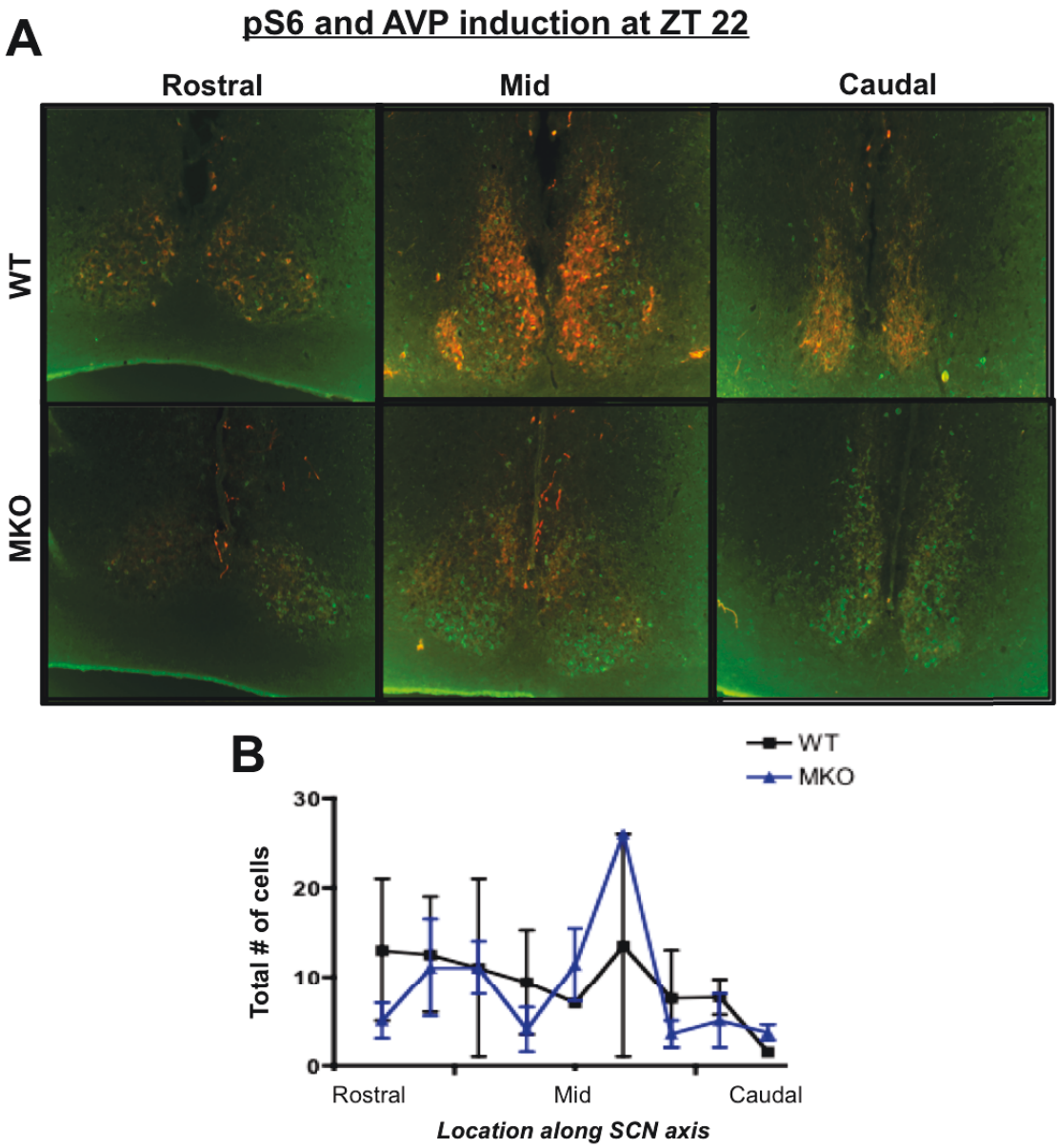


FIGURE 9: There is no difference between the number of AVP cells that are pS6 positive in WT and MKO animals during the early night.
A) AVP co-stained with pS6 following 15 mins of light in the SCN of WT and MKO animals at ZT 22. 25 um SCN sections shown. B) Rostral to caudal distribution of co localized AVP and pS6 positive cells at ZT 22 in the SCN of WT and MKO animals.

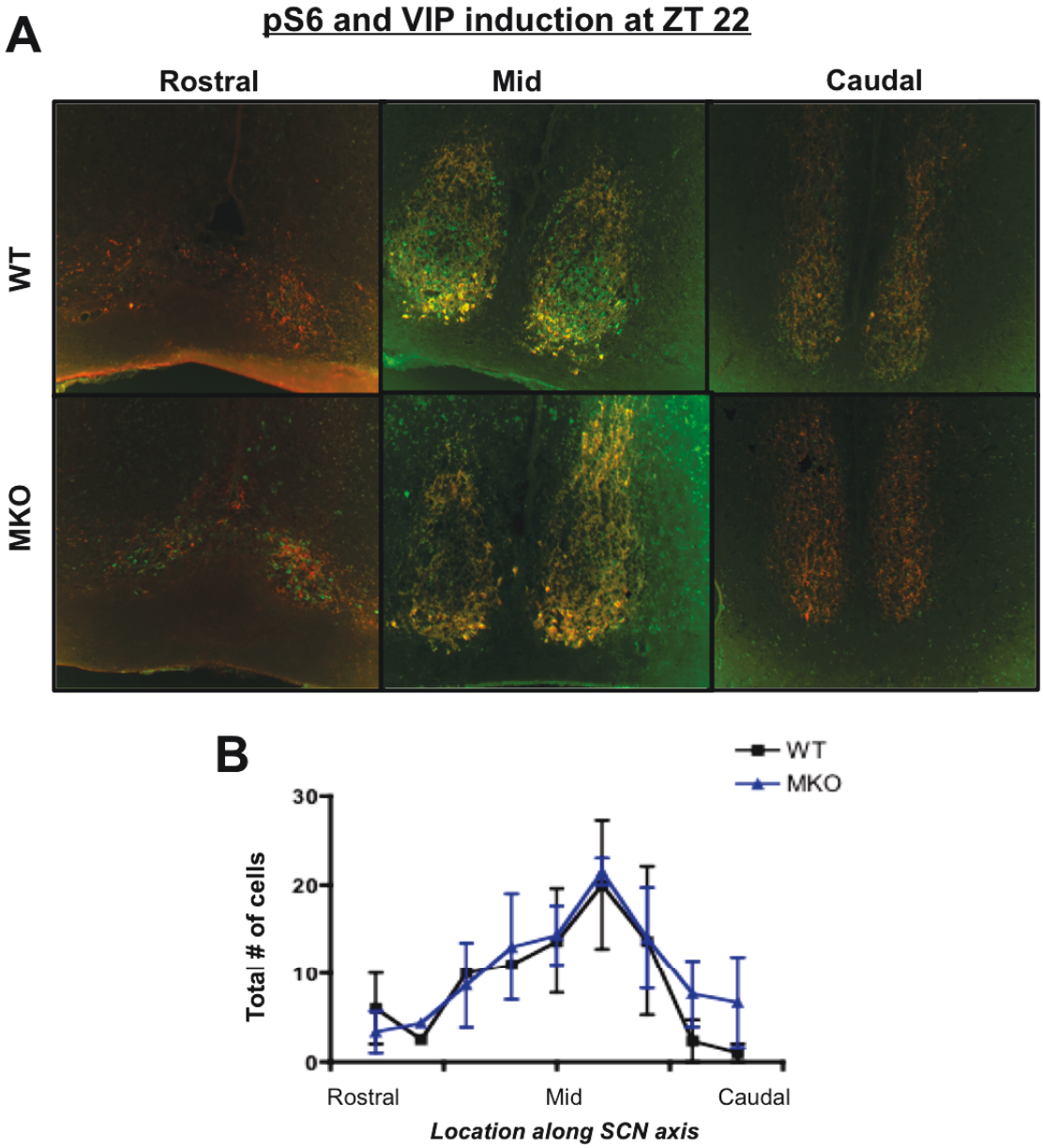


FIGURE 10: Light stimulates a similar number of VIP cells in the SCN that are pS6 positive in WT and MKO animals during the early night.
A) VIP co-stained with pS6 following 15 mins of light in the SCN of WT and MKO animals at ZT 22. 25 um SCN sections shown. B) Rostral to caudal distribution of co localized VIP and pS6 positive cells at ZT 22 in WT and MKO SCN

Melanopsin is important in a subset of ipRGCs that predominantly project to the SCN for light-mediated c-fos expression in the SCN.

In addition to the SCN, ipRGCs project to a wide variety of brain nuclei. To determine whether the SCN was playing the primary role in the light induction of c-fos, I took advantage of two mouse lines in which the majority of ipRGCs are ablated, except for the ones that predominately target the SCN. The ablated ipRGCs belong to the population of ipRGCs that express the transcription factor, Brn3b. These Brn3b-positive ipRGCs are ablated *in vivo* using the cre-loxP method, where an ipRGC-specific melanopsin-Cre drives genetic recombination, leading to the expression of diptheria toxin in these cells. The Brn3b-negative ipRGCs are the only ipRGCs that survive in these animals (mouse fully described in Chen *et al* 2011). I used two variations of these animals: the "Brn3bzDTA; Cre/+" in which one functional copy of melanopsin was present, and the "Brn3bzDTA; MKO" in which melanopsin absent in those ipRGCs (this mouse is homozygous for melanopsin-cre). Therefore, the only difference between these animals with only Brn3b-negative ipRGCs is the presence or absence of melanopsin.

In the presence of melanopsin, there is a slight difference in c-fos expression in the rostral region of the SCN during the early night versus the late night (Figure 11A). Whereas the total number of c-fos positive

cells tends to differ between the early night and late night (Figure 11B), the immunostaining for c-fos along the rostro-caudal axis in SCN is the same during the early night compared to the late night (Figure 11C). In the absence of melanopsin, when the Brn3b-negative ipRGCs rely solely on the light input from rods and cones, there are more c-fos positive ipRGCs in the rostral SCN in response to light during the early night as compared to the late night (Figure 12A). Whereas no difference exists in the total number of c-fos positive cells between the early night and late night (Figure 12B), there fewer c-fos positive cells mid-caudal SCN in the absence of melanopsin (Figure 12C).

To determine whether melanopsin was playing a role in light response in the SCN, I compared c-fos induction in Brn3bzDTA; MKO and Brn3bzDTA; Cre/+ animals during the early night and late night. I expect that if melanopsin was important in the SCN-projecting ipRGCs for the c-fos expression pattern and not having a secondary effect through another non-SCN target, then the results observed in the MKO animals with all ipRGCs should be true when only the Brn3b-negative ipRGCs remain and melanopsin is removed. During the early night, there are significantly fewer c-fos positive SCN cells in the absence of melanopsin when only the Brn3b-negative ipRGCs remain (Figure 13A-B). The difference was observed in the mid-caudal region of the SCN. During the late night however, the number of c-fos positive SCN cells is similar in the absence or presence of melanopsin (Figure 13C-D). These

data are consistent with my findings that melanopsin is important for light dependent induction of c-fos in the SCN during the early night but not the late night. The Brn3b-negative ipRGCs are sufficient to mediate this effect.

Rods are not necessary for light driven c-fos induction in the SCN.

Mice that contain functional rods, non-functional cones, and no melanopsin in ipRGCs, exhibit normal circadian photoentrainment, which suggests that rods play a role in such SCN-mediated behavior (Altimus *et al.,* 2010). To determine whether rods are important for the light-induced c-fos expression in the SCN, I used the H-red; RKO mouse (Human-red cone knock in; rod knockout). In this mouse, there is a knock-in of a human red cone opsin cDNA in the locus of the green cones on the X-chromosome (Smallwood *et al.,* 2003). This shifts the maximal activation of green cones towards red (~560 nm) away from their normal 500 nm and away from 480 nm which activates melanopsin, which potentially allows us to use specific wavelengths of light to activate the each photoreceptor. Melanopsin, however, is less sensitive than rods and cones and therefore requires a higher intensity of light to induce a photic response. Although melanopsin responds to blue light (480nm), it is very likely that due to the intensity of light required to activate melanopsin, as well as the broad range of spectral sensitivity amongst cones and rods, that blue light activates rods and cones in addition to

melanopsin. Therefore in this mouse a light stimulus of a high enough intensity may similarly activate all photoreceptors. This caveat was later realized. Although I was unable to precisely activate melanopsin with the blue light, I obtained access to the H-red mouse, which was mated to the RKO (rod knockout animal- (Altimus *et al., 2010*) in our lab by Alan Rupp (another graduate student)).

Therefore, with the use of a 15 minutes, blue-shifted short wavelength light stimulus, my hypothesis is that if in addition to melanopsin, rods are important for c-fos expression in the SCN, then there should be a small number of cells with c-fos expression in both the early and late night. I predict that there will be at least some melanopsin-driven c-fos expression during the early night and possibly no c-fos expression during the late night is rods are primarily responsible for late night light responses. In comparison to WT animals stimulated with white light (moderately bright-500 lux), blue light stimulates c-fos expression in SCN cells of H-red; RKO mice in both the early and the late night (Figure 14).

These data indicate that rods are not necessary for WT-like expression of c-fos in the SCN at either the early night or the late night. Additionally, since melanopsin is not required for WT-like c-fos expression in the SCN during the late night, cones may be involved in the late night light response.

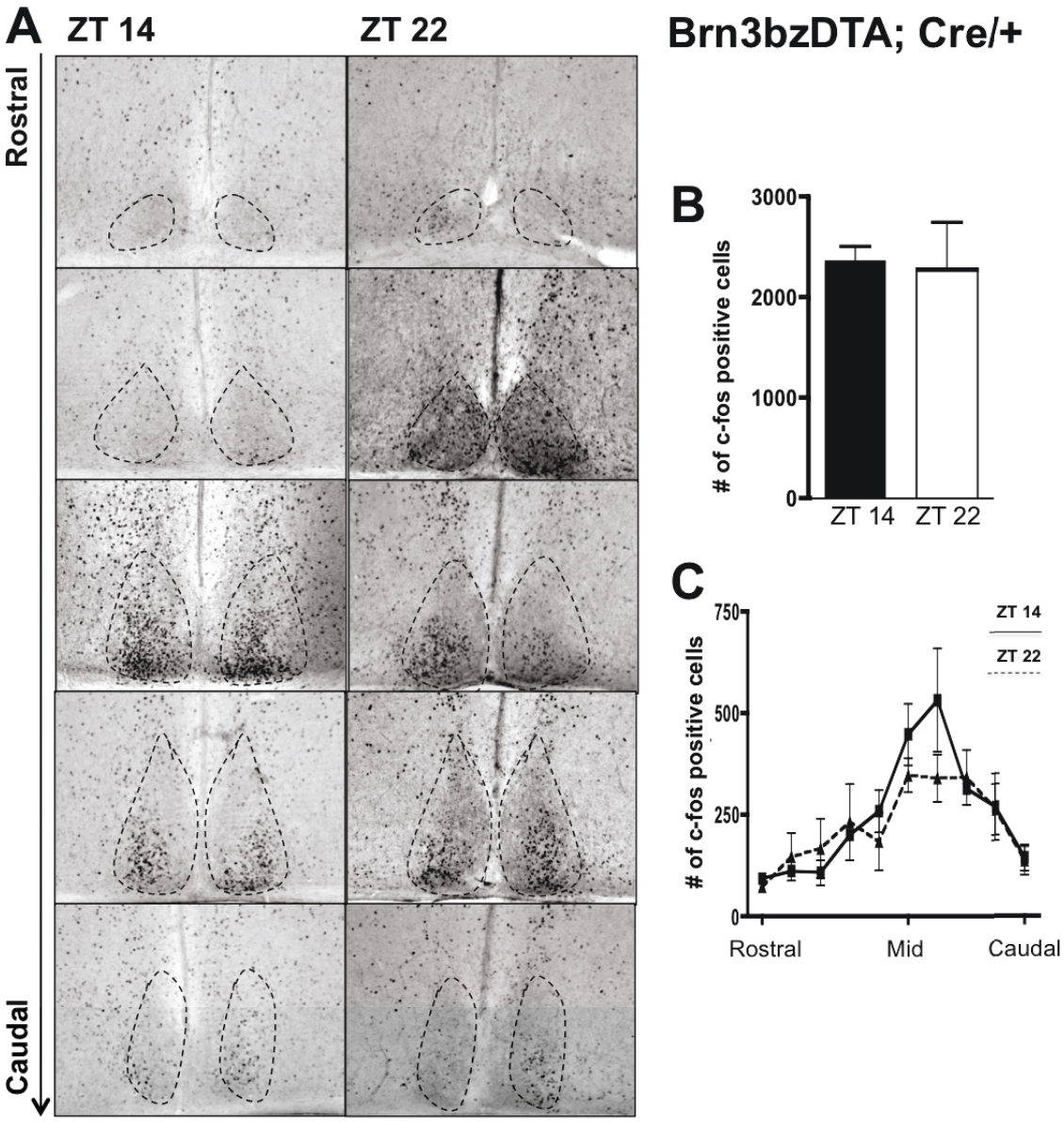


FIGURE 11: Time of light exposure has little effect on c-fos induction in mice that continue to express melanopsin and only have Brn3b-negative ipRGCs.
A) c-fos induction by 15 mins of light in Brn3bzDTA; Cre/+ SCN at ZT14 and ZT 22. 50 um rostral to caudal sections of the SCN. Every second section shown. B) No light pulse control. C) Quantification of total c-fos positive cells at ZT 14 and ZT 22 in the SCN of Brn3bzDTA; Cre/+ animals. D) Rostral to caudal distribution of c-fos positive cells at ZT 14 and ZT 22 in the SCN of Brn3bzDTA; Cre/+ animals.

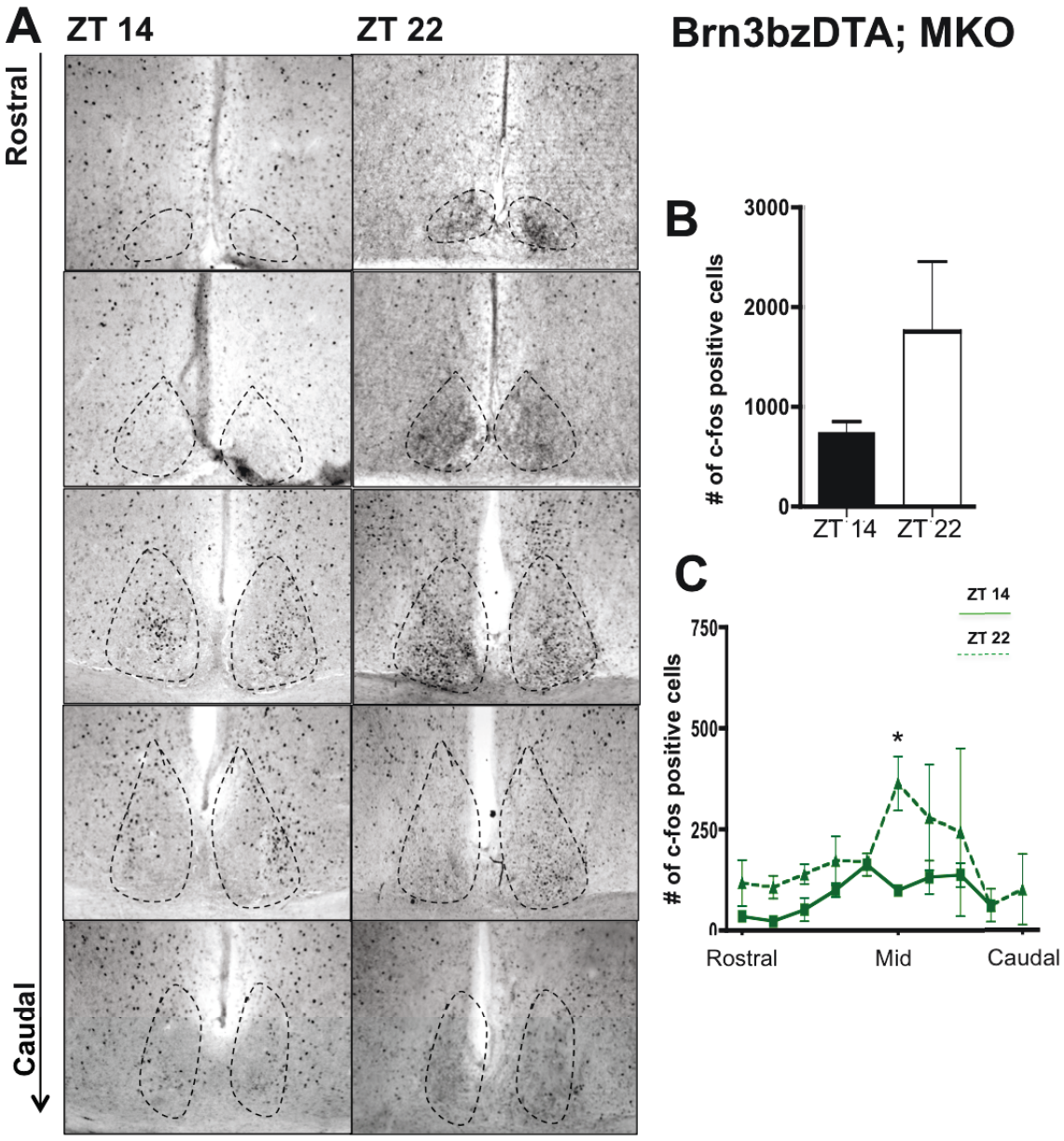


FIGURE 12: Late night light exposure drives c-fos induction through most of the SCN even in the absence of melanopsin expression in Brn3b-negative ipRGCs.
A) c-fos induction by 15 mins of light in the SCN of Brn3bzDTA; MKO animals at ZT14 and ZT 22. 50 um rostral to caudal sections of the SCN. Every second section shown. B) Quantification of total c-fos positive cells at ZT 14 and ZT 22 in the SCN of Brn3bzDTA; MKO animals. C) Rostral to caudal distribution of c-fos positive cells at ZT 14 and ZT 22 in the SCN of Brn3bzDTA; MKO animals. Statistical analyses: two-tailed student's t-test. P-value: *P<0.05.

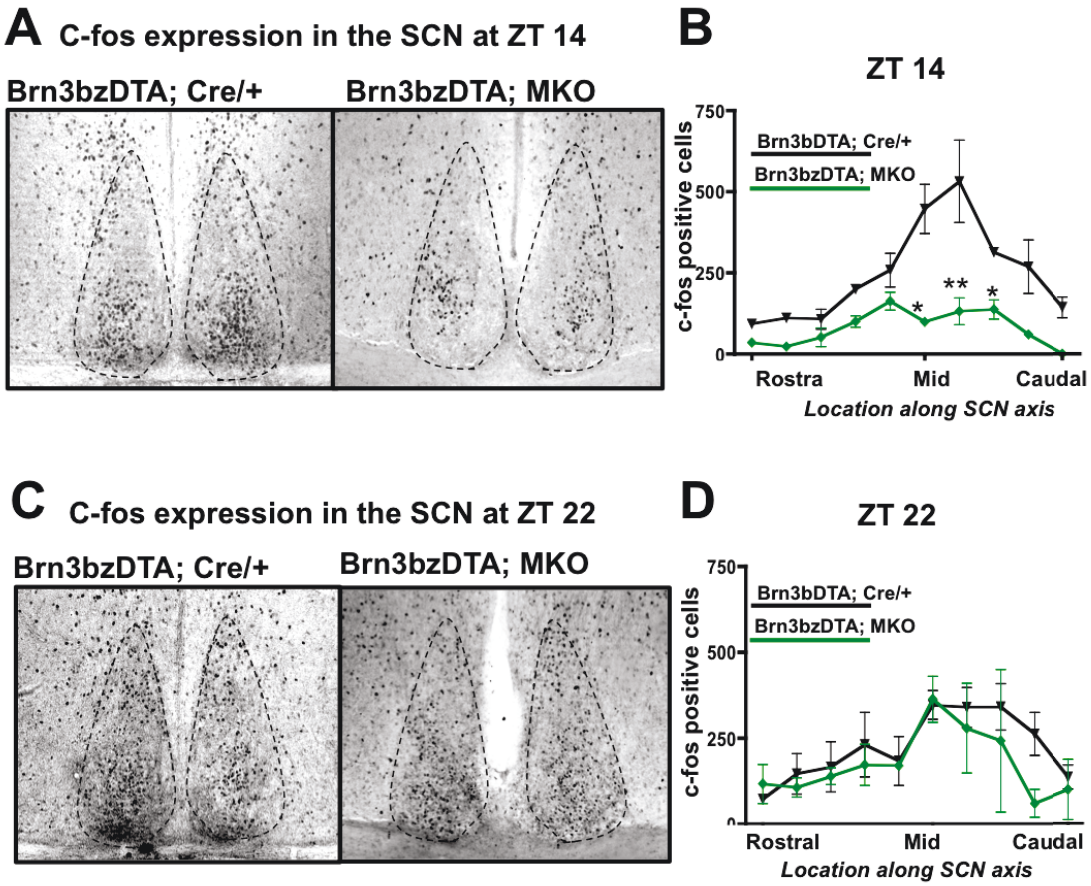


FIGURE 13: Melanopsin expression by Brn3b-negative ipRGCs is necessary for c-fos induction in the early night but not the late night.
c-fos induction by 15 mins of light in the SCN of Brn3bzDTA;Cre/+ and Brn3bzDTA; MKO animals at ZT14. 50 um mid-SCN section shown. B) Rostral to caudal distribution of c-fos positive cells at ZT 14 in the SCN of Brn3bzDTA;Cre/+ and Brn3bzDTA; MKO animals. C) c-fos induction by 15 mins of light in the SCN of Brn3bzDTA;Cre/+ and Brn3bzDTA; MKO animals at ZT 22. 50 um mid-SCN section shown. D) Rostral to caudal distribution of c-fos positive cells at ZT 22 in the SCN of Brn3bzDTA;Cre/+ and Brn3bzDTA; MKO animals. Statistical analyses: Two-way ANOVA. P-values: *P<0.05, **P<0.01.

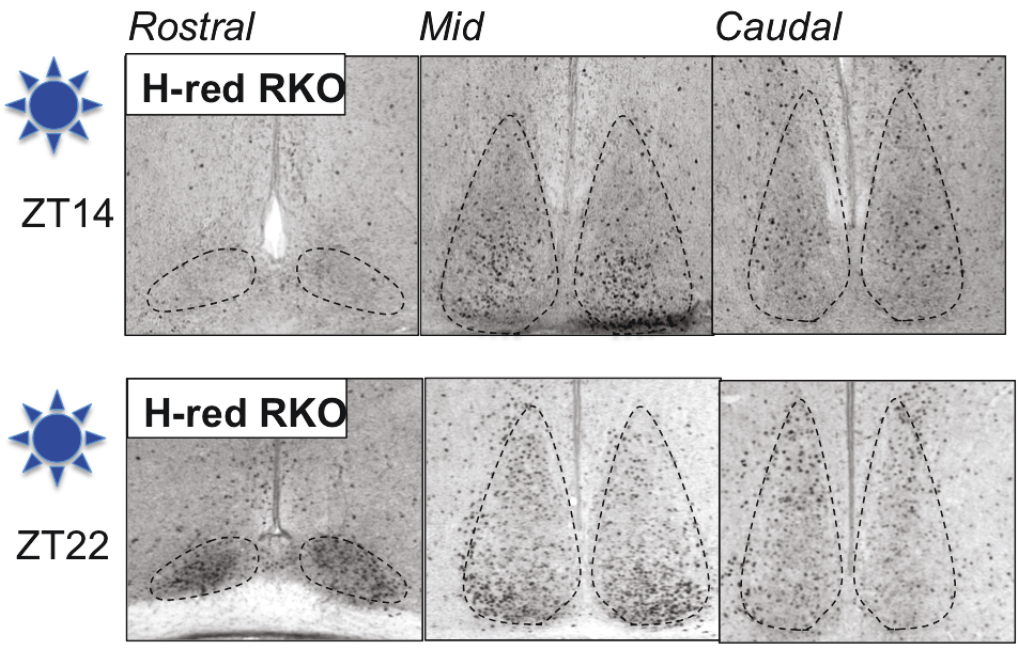


FIGURE 14: Though important for circadian photoentrainment, rods are not necessary for a WT-like pattern of c-fos activation in the SCN during the early and late night.

15-minutes of blue shifted light was used to preferentially activate melanopsin in mice that contained rods which no longer respond to light and human red cone knock-in. 50um representative sections shown from the rostral, mid and caudal SCN.

DISCUSSION AND CONCLUSIONS

SCN light activation pattern: significance and implications

I demonstrated that melanopsin is important in how the SCN processes light information along the rostro-cadual axis of the SCN and across the night. I noticed two types of effects on c-fos induction; these involve time of exposure differences across the SCN and melanopsin-mediated differences during the early night. These findings may be significant for time-of-day specific modulation of an array of biological processes such as hormonal release and cyclic gene expression. More extensive studies should be conducted to determine whether the c-fos and pS6 act in a complementary way during light activation of the SCN.

While we know that light induces circadian phase shifting based on the time of day at which the light pulse is administered, we do not understand the physiological significance of direction or magnitude of the phase shift (Daan 2000; Mistlberger and Antle 2011). From my work, it is clear that there are differences in which cells are activated by light in the SCN during the early versus late night. Therefore, future studies could focus on understanding which cells are activated and what kinds of synaptic connections these cells make with both the retina as well as extra-SCN targets.

Regional variation across the SCN seems to be potentially important for mediating responses to brief early-night light exposure,

particularly in the presence of melanopsin. The same regional variation is true for late night light responses where instead of melanopsin, extrinsic light input from cones, may mediate the c-fos expression in the SCN. The regional and temporal variation in light induced expression of c-fos in the SCN could be important for a variety of circadian-modulated behaviors. For instance, the level of the homeostatic drive at different times of the night, which is known to drive sleep, a circadian-modulated behavior may be regulated by light input to specific regions of the SCN at specific times of the day and night (Saper *et al., 2005*).

Clinical applications for melanopsin-regulated, circadian-dependent light activation of the SCN

Light therapy treatments for illnesses such as seasonal depression and sleeping disorders, and for jet lag recovery, use the knowledge that light presented at a specific time of the day can shift the circadian clock to either a later or earlier time (Sack *et al.,* 1990; Rosenthal *et al.,* 1990). However, the scientific basis for this method is requires revision as experiments were conducted in constant darkness and thus, is not reflective of the typical light-dark environment people experience. The research discussed in this chapter of my thesis uncovers the necessity to re-evaluate the current methods used in treating patients with light. Furthermore, with future study, light treatment options can focus on

activating specific brain regions, which can result in more effective treatment for light-regulated circadian disorders.

Chapter 3: The effects of day length and irregular light schedules on circadian rhythms

INTRODUTION

In a society filled with major technological advances and a growing entertainment industry, the chance of exposure to light at night is very likely. To most, the opportunity to be entertained around the clock, or even work an extra shift at night is welcomed. However, this opportunity is not without consequence; light exposure outside of earth's natural day can lead to severe health effects in most people. While some nighttime light exposure is unavoidable, many people in our society are being intentionally exposed to light at night. While light exposure at night usually causes shifts in the phase of the circadian clock, the duration and intensity of the light are relevant variables, as these factors underlie the severity of the body's response to aberrant light.

Alignment of circadian rhythms with light-dark environment of earth is necessary for normal physiological function. The intrinsic period in mice is approximately 24 hrs and matches the earth's day-night cycle. While an entrained circadian rhythm is most common and the landmark for what is considered normal, there are other types of circadian rhythms. The intrinsic "free-running" circadian period is the endogenous period of the circadian clock but it is observable only in the absence of light input, whether from the environment or a lack of retinal photo-transducers of light to the brain. This intrinsic period in mice is approximately 23.5 hrs. A third type of circadian period is distinctly abnormal. When the circadian pacemaker, the SCN, is not functioning

normally, an arrhythmic circadian rhythm is commonly observed. In this case, the circadian clock does not respond to the light from external environment or have a steady internal rhythm of its own. And fourth, a lengthened circadian rhythm is observed in mice under constant light conditions (LL). The period under constant light obeys Aschoff's rule, which states that the circadian period (tau) increases with light intensity in nocturnal animals (Aschoff 1960; Aschoff 1979). While period lengthening experiments are usually conducted in constant bright light conditions, this chapter will discuss other types of environments that can lead to a lengthened period, which is approximately 25 hrs in mice.

Of all these types of non-entrained circadian rhythms, only period lengthening is light regulated. The effect of environment light on driving period lengthening is not well understood. Light exposure at night is very disruptive to circadian rhythms; however, the entrainment of the circadian period with light-dark environment is not disrupted. Previous studies have documented that period lengthening also occurs in the T7 cycle (3.5hrs light: 3.5hrs dark) although the physiological relevance of these findings were not fully explained (LeGates *et al.,* 2012). Period lengthening in the T7 cycle leads me to consider that period length is not solely a function of the increasing intensity of constant light, but that like the T7 cycle, constant light is a disruptive and un-entrainable light environment that leads to period lengthening. The questions that remain are: what kinds of disruptive environments result in period lengthening?

How can light drive both a 24-hour circadian period and a lengthened period?

Previous studies have shown constant light information is likely to be mediated partially by melanopsin photoreception and partially by rod/cone signaling. When melanopsin is absent in ipRGCs, partial lengthening of the circadian period in LL is observed (Ruby *et al.,* 2002). Therefore, I investigate the role of melanopsin in period lengthening in disruptive light-dark environments to further our understanding of this circadian rhythm. There is also clinical significance to understanding the role of melanopsin in period lengthening, as blue light, which activates melanopsin, widely used for treatment of circadian dysfunctions. Therefore, if these treatments are incorrectly used, harmful effects such as period lengthening could occur.

METHODS

All mice were of a mixed background (BL/6;129SvJ). Male animals that were used in the behavioral analyses were aged between 3 and 12 months. Animals were housed and treated in accordance with NIH and IACUC guidelines, and used protocols approved by the Johns Hopkins University Animal Care and Use Committees.

Wheel-running activity

Mice were placed in cages with a 4.5-inch running wheel, and their activity was monitored with VitalView software (MiniMitter). Analyses of wheel running activity were calculated with ClockLab (Actimetrics). 500 lux light intensity (moderately bright-office lighting) was used in all expermients. Mice were initially placed under 12:12 LD or 16:8 LD cycle for 2 weeks before any jet lag experiments.

RESULTS

A variety of light cycles can induce period lengthening.

Period lengthening occurs under a variety of irregular light-dark environments. In a light-dark cycle comprised of 3.5 hours of light and 3.5 hours of dark, also referred to as T7-cycle, WT mice exhibit a lengthened circadian period (Figure 15 A, adapted from LeGates 2012). Period lengthening occurs during constant bright light exposure (Aschoff 1979; Ruby *et al.,* 2002; Figure 15 B). Mice maintained under a T6 (2-hours dark and 4-hours light) cycle, also exhibit a lengthened period (Figure 15 C and D). These data allow me to conclude that period lengthening occurs in a variety of disruptive light environments.

To investigate why period lengthening occurs under T7, LL and T6 cycles, I studied circadian rhythms under a variety of disruptive light-dark environments. Specifically, I looked to determine whether period lengthening in the T7 cycle was the result of insufficient darkness (3.5 hrs) to entrain the circadian clock. During the T7 cycle, there are 12 total hours of darkness and the total amount of sleep is normal (LeGates 2012). Therefore, I placed WT mice under a LD paradigm with 20 hrs of light and 3 hrs of dark. WT mice display normal photoentrainment under these conditions even though they were exposed to a shorter period of darkness that are animals under the T7 cycle. These data suggest period lengthening under the T7 cycle is not a result of the reduced length of

darkness, but rather the distribution of the dark and light periods throughout the day (Figure 16 A).

Since a consolidated LD cycle, as seen in the 20:3 LD cycle, results in normal circadian photoentrainment, I disrupted the night of a typical 12:12 LD cycle with chronic daily light exposure. My aim was to determine if disrupting the night, thus leading to more light and dark periods within 24 hrs, would lead to a lengthened period. In the first experiment, I exposed WT mice to 15 mins of light during the early part of the night (ZT14), which is known to phase shift the circadian period. While I did observe phase shifting and activity suppression during the light pulse, this LD paradigm did not lead to period lengthening (Figure 16 B). I also observed decreased activity prior to the light pulse; this reduction could be a result of the phase shift.

Both the T7 and T6 LD cycles, have 7 or 8 periods of either light or dark in a 24 hr period, but these periods of light and dark are evenly distributed across the day. The disruption itself may be the cause of period lengthening in these animals. To test this possibility, I disrupted the night portion of a 12:12 LD cycle with pulses of light of varying durations and at varying times during the subjective night period. The following schedules were used: 3, 15 mins light pulse at night (12hrs L: 2hrs D: 15-mins L: 3.75 hrs D: 15-mins L: 4hrs D: 15-mins L: 1.75hrs D) or 3, 1 hour of light pulse at night (12hrs L: 2hrs D: 1hr L: 3 hrs D: 1hr

L: 3hrs D: 1hr L: 1hr D). In both of these light cycles, there were 8 periods of either light or dark in a 24 hr period. I found that neither the multiple 15-minute nor multiple 1-hour light exposure at night produced a lengthened circadian period. Significant light-driven activity suppression (known as masking) did occur as a result of the light exposure (Figure 16 C and D). From these data, I conclude that while multiple aberrant light pulses at night leads to acute masking effects, period lengthening does not occur.

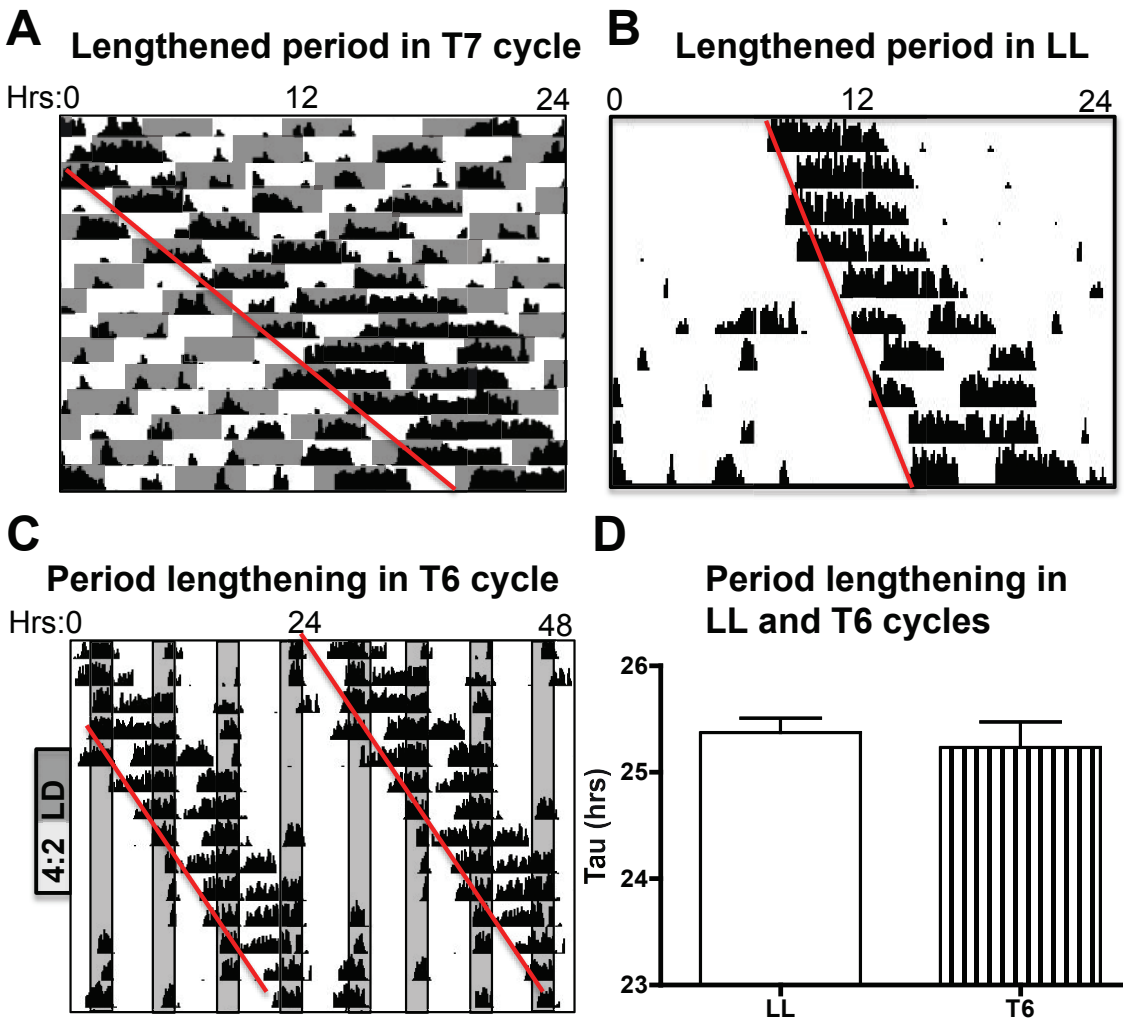


FIGURE 15: Period lengthening occurs under a variety of irregular light-dark environments.

A) Period lengthening in a WT animal under T7 LD cycle (LeGates *et al* 2012). B) Period lengthening in a WT animal in constant light (LL) . C) Period lengthening in the T6 LD cycle. D) Quantification of period lengthening in LL and T6 LD cycle. Red lines indicate lengthened period.

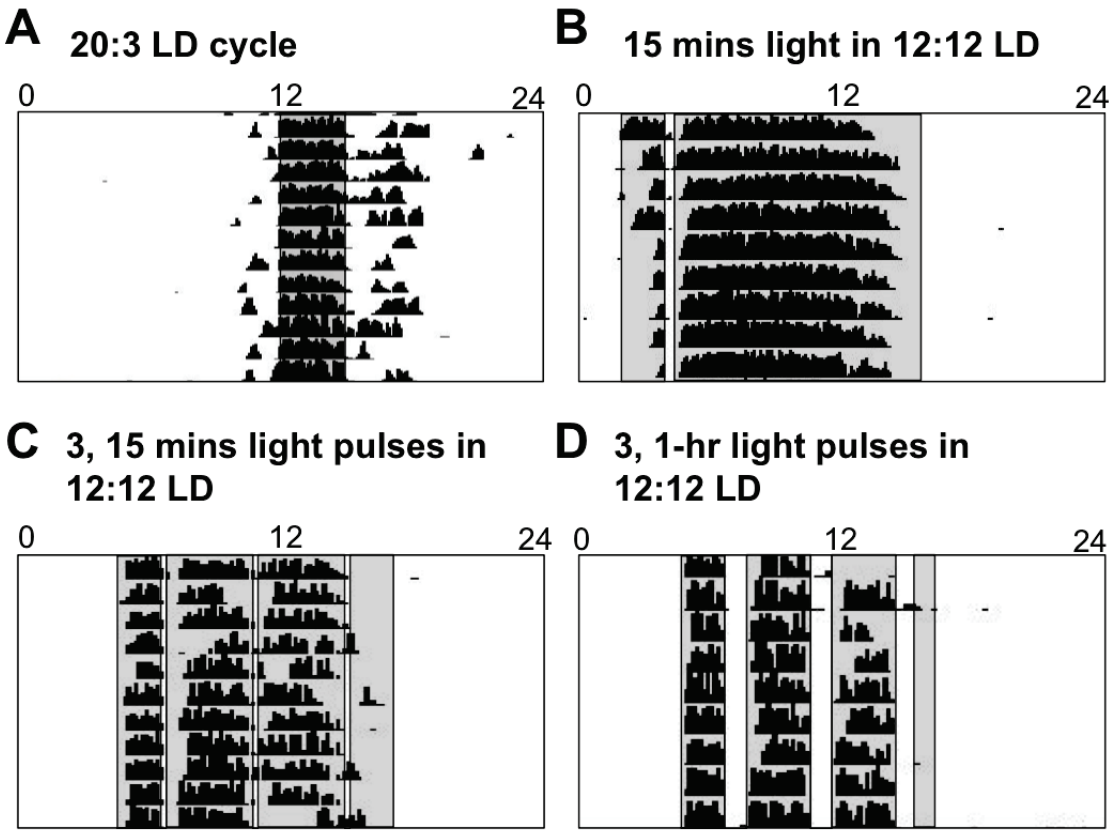


FIGURE 16: Not all disruptive light-dark environments lead to period lengthening. A) 20:3 LD cycle in WT animals. B) 15 mins of light exposure 2 hrs after dark onset in a 12:12 LD cycle in WT animals. C) 15 mins of light exposure 2 hrs, 5 hrs, and 10 hrs after dark onset in a 12:12 LD cycle in WT animals. D) 1 hour of light exposure 2 hrs, 5 hrs, and 10 hrs after dark onset in a 12:12 LD cycle in WT animals.

Prior light history influences the magnitude of period lengthening in the absence of melanopsin.

For many years, scientists have investigated the role of melanopsin in light mediated circadian responses. A deficit in period lengthening is among the few known roles for melanopsin (Ruby 2002). However, analysis of period lengthening has generally been for the purpose of compliance with Aschoff's rule that dictates the period of a nocturnal animal will increase with light intensity in constant light conditions (Aschoff 1979; Ruby 2002). I further investigated the role of melanopsin in period lengthening to determine what period lengthening is as well as what role may melanopsin play in this behavior.

Period lengthening phenotypes have been observed in response to either gradual increase in the intensity of constant light or following constant darkness conditions. In both of these conditions, either darkness or light prior to the constant light condition that induced the lengthened period. Therefore, I tested whether the light environment prior to LL affects the period length in LL and whether melanopsin expression by ipRGCs was important for responding to a change in the LD environment. WT mice released into LL following display significantly lengthened their period in LL. MKO animals do not. They maintain almost the same period length in LL as the one they exhibit in 12:12 LD (Figure 17 A and B). To determine if the cyclical nature of the 12:12 LD

cycle affect the occurrence and magnitude of period length, I removed all light stimuli by placing mice in constant darkness (DD). Following DD, WT animals lengthen their period similar to what is observed following 12:12 LD (Figure 17 C and D). Although not so strongly as WT animals, MKO animals lengthen their period in LL following DD (Figure 17 C and D). That lengthened period as the transition from LL to DD is significantly longer than the lengthening hey exhibit hen they transition from 12:12 LD to LL (Figure 17 E). Therefore, I conclude that in the absence of melanopsin, the rod/cone input from the outer retina to period lengthening depends on the previous light environment. These data make a strong case for the role of prior history of light environment on the behavior of animals that rely on rod and cone input to ipRGCs.

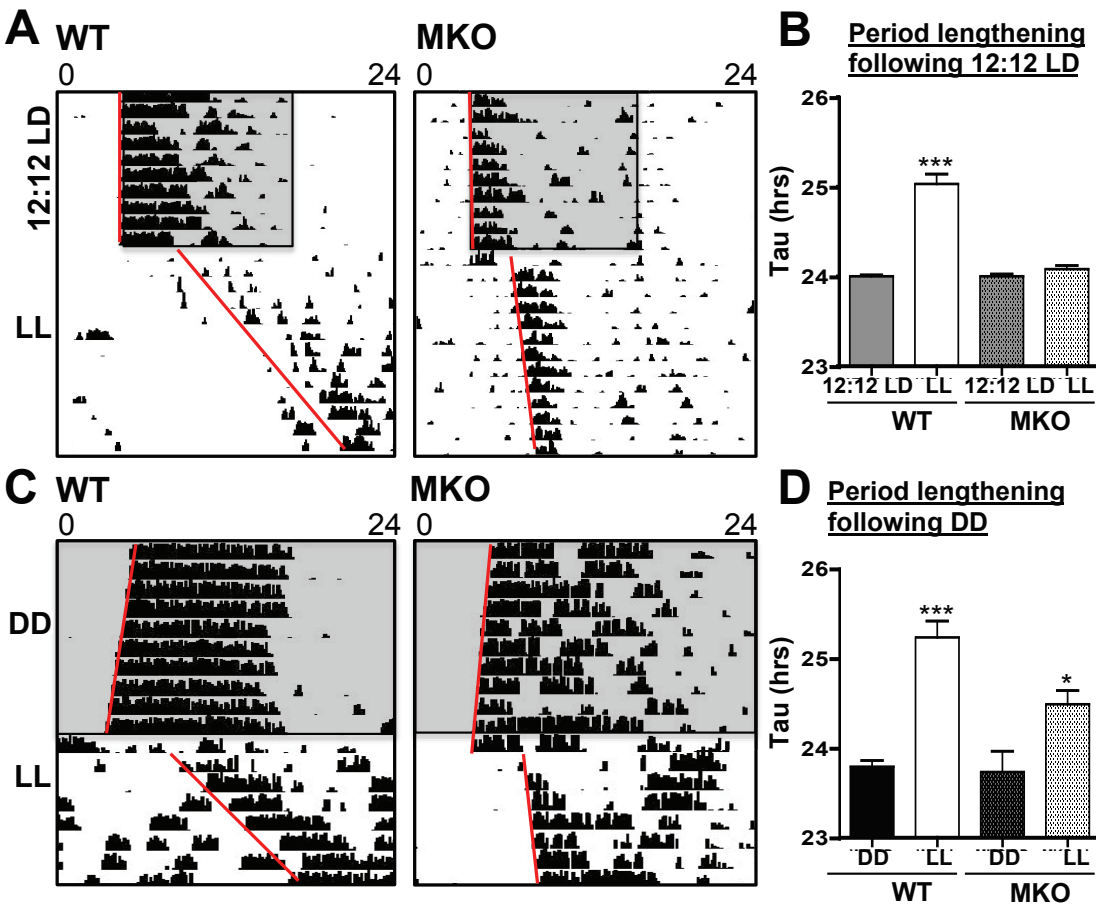


FIGURE 17:
Melanopsin is necessary for period lengthening in LL following 12:12 LD.
A) Period lengthening in a WT and MKO animals under LL following 12:12 LD. B) Quantification of the period (tau) in 12:12 LD and LL in WT and MKO animals. C) Period lengthening in a WT and MKO animals under LL following DD. D) Quantification of the period (tau) in DD and LL in WT and MKO animals. E) Period length in LL following DD and 12:12 LD. Re-plotted from B and D. Red lines indicate main differences in behavior observed. Statistical analysis: two-tailed student's t-test. P-values: *P<0.05, **P<0.01, ***P<0.001.

Prior light history influences the intrinsic free-running period in the absence of melanopsin.

To further understand how prior light exposure affects circadian rhythms, I determined how the intrinsic circadian rhythms are altered after period lengthening. When either WT or MKO animals are exposed to constant darkness following a 12:12 LD cycle, they exhibit a free-running period of around 23.5 hrs (Figure 18 A and B). Since MKO animals display two different period lengths in LL following 12:12 LD and DD, I investigated whether the intrinsic rhythm could be altered by the prior light environment. I analyzed the intrinsic period in DD, following LL, with prior light exposure in either a 12:12 LD or 20:4 LD cycle. In both conditions, WT mice exhibit a shortened free-running period in DD of ~23.5 hrs (Figure 18 C-F). MKO animals in contrast exhibit small differences in the free-running period (Figure 18 C and D). They display no difference following 20:4 LD then LL, and maintained the circadian period they exhibited in LL (Figure 18 E and F). These results show that melanopsin plays a crucial role in transitioning between different types circadian rhythms and that prior light history is an important factor in driving this behavior.

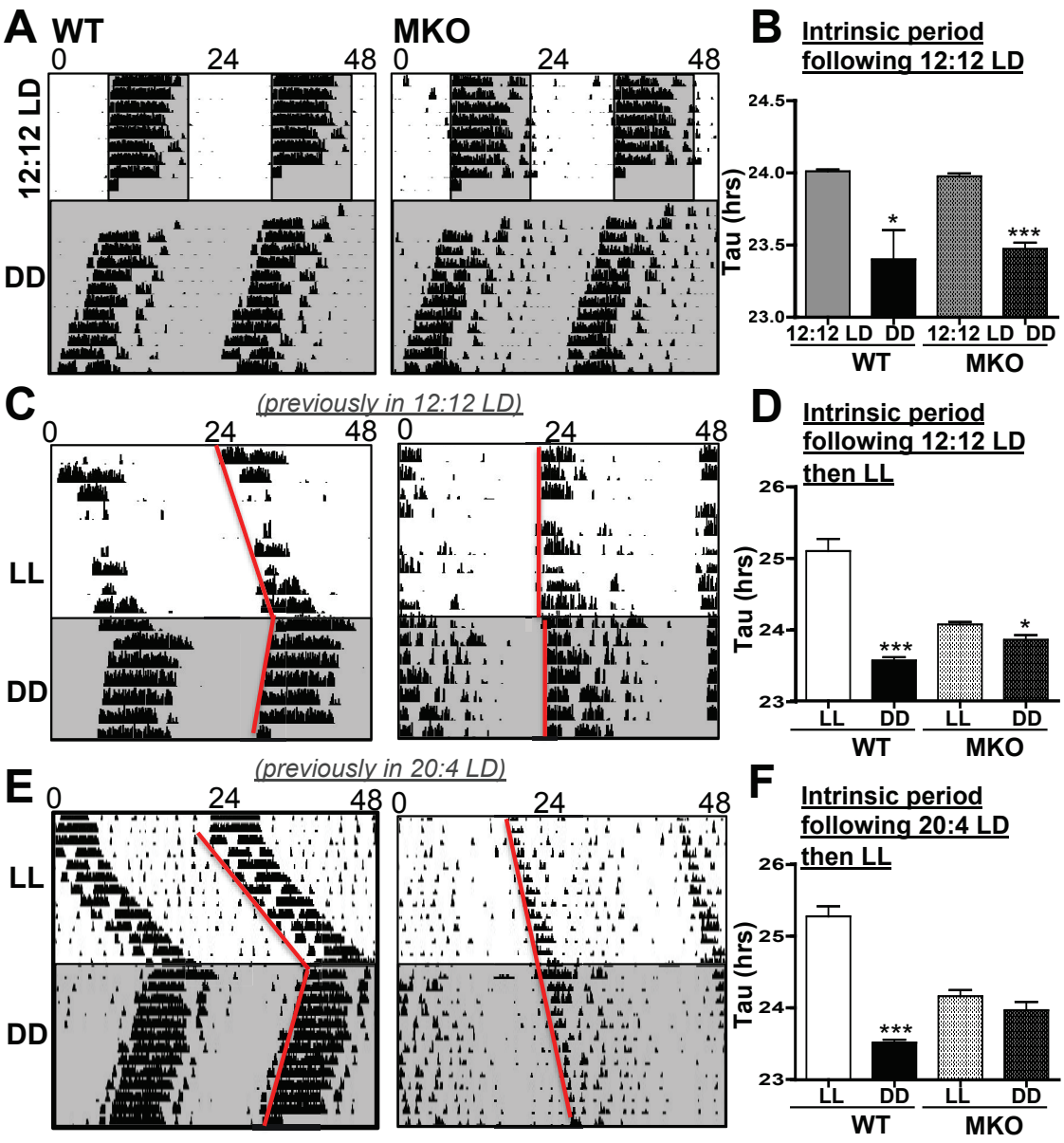


FIGURE 18: Melanopsin is necessary to recover the intrinsic free-running period in DD following LL.

A) Free-running period in DD following 12:12 LD. B) Quantification of period in (A). C) Free-running period in DD following LL and 12:12 LD. D) Quantification of period in (C). E) Free-running period in DD following LL and 20:4 LD. F) Quantification of period in (E). Red lines indicate main differences in behavior observed. Statistical analysis: two-tailed student's t-test. P-values: *P<0.05, ***P<0.001.

Is period lengthening always dependent on the presence of melanopsin?

MKO animals exhibit period lengthening deficits in LL, but it is unclear whether these deficits are a function of the lack melanopsin or the nature of light environment. Therefore, I investigated whether disruptive light dark environments other than LL that caused period lengthening in WT animals would also yield a deficit in period lengthening in MKO animals. When a T6 cycle followed a 12:12 LD cycle, both WT and MKO animals exhibited lengthened circadian periods (Figure 19 A and B). Two variations of the phenotype are observed where both WT and MKO animals either exhibit a moderately lengthened period with activity in all aspect of the dark portions of the day (A) or a lengthened period similar to that observed in LL (B).

The "jet lag" paradigm is a paradigm in which the light dark cycle is shifted earlier or later by 6-hrs. In a 6-hr shifted 16:8 LD jet lag paradigm, WT animals quickly adjust to the changes in the light dark environment (Figure 20 A, also discussed in chapter 4). Most MKO animals also adjust normally to the jet lag paradigm, but a few exhibit a lengthened period (Figure 20 B). Furthermore, in MKO animals that only contain the SCN-projecting ipRGCs (Brn3bzDTA; MKO animals, discussed in chapter 4), period lengthening occurs in the 6-hr shifted 16:8 LD cycle (Figure 20 C). Whether all ipRGCs are present or only the

ipRGCs that predominantly target the SCN, period lengthening is more likely to occur in the absence of melanopsin of melanopsin in these jet lag paradigms. Melanopsin in ipRGCs, however, is necessary for period lengthening in LL whether all ipRGCs are present or only the ipRGCs that predominantly target the SCN (Figure 20 D). To further test if period lengthening, driven in melanopsin-independent manner, was more than a behavioral output of the jet lag paradigm, I observed photoentrainment in LD cycles in which the days were lengthened. I found that while both WT and MKO animals entrain to a 20:4 LD cycle, disrupting the night with a 1 hr pulse each day can lead period lengthening in MKO animals (Figure 21). Therefore, period lengthening is driven in both a melanopsin-dependent and melanopsin-independent manner in.

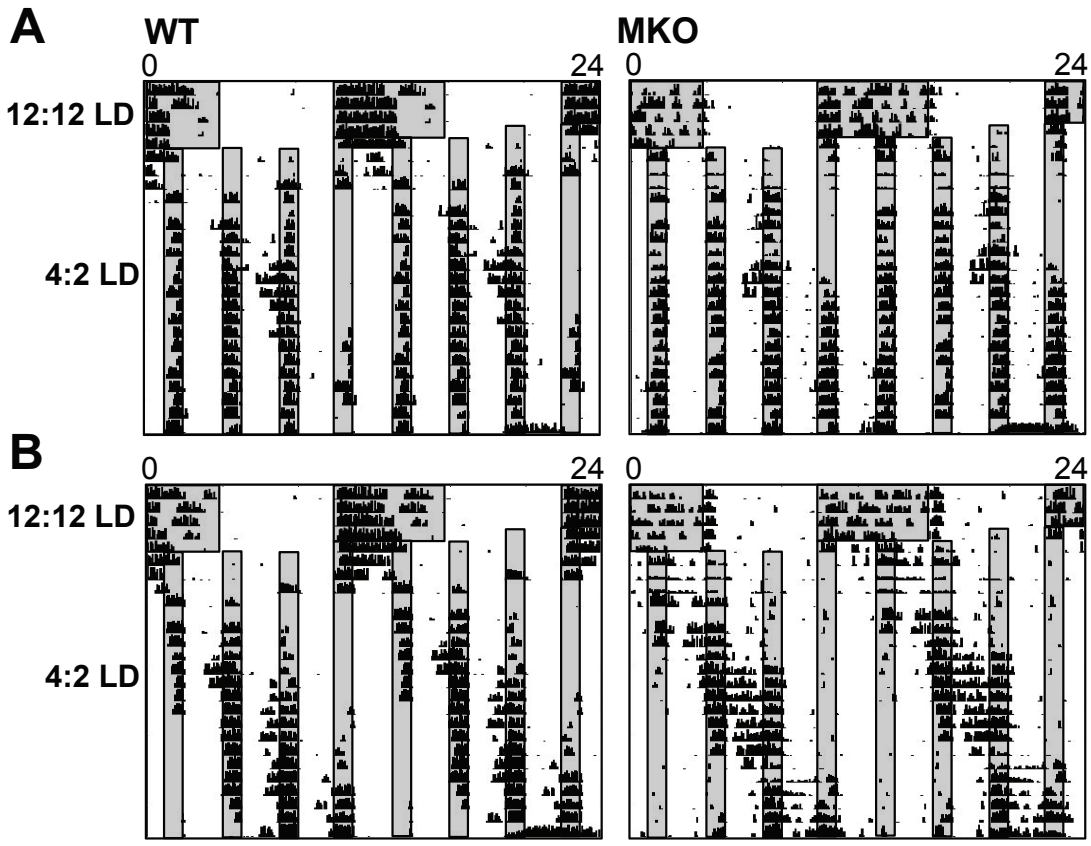


FIGURE 19: Period lengthening occurs in the T6 LD cycle in a melanopsin independent manner.

A) Phenotype-1 (50%) of WT and MKO animals in T6 cycle (4:2 LD). B) Phenotype-2 (50%) of WT and MKO animals in T6 cycle (4:2 LD).

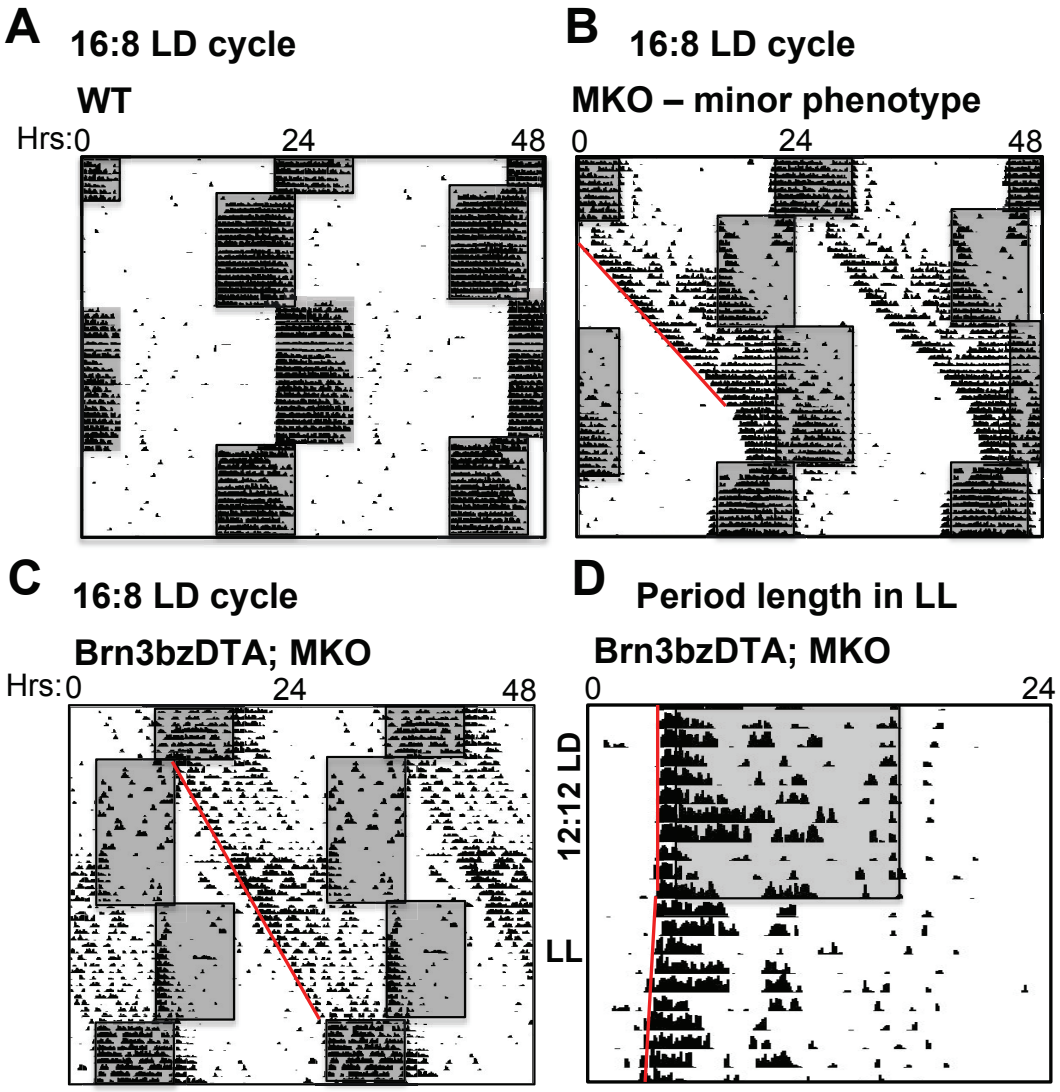


FIGURE 20: Period lengthening occurs as a result of disruptive changes in the light-dark environment in a melanopsin independent manner.
A) WT animals entrain normally to 6-hr advances and delay in the 16:8 LD cycle. B) 3/8 MKO animals exhibit lengthened circadian period in a 6-hr advanced and delayed 16:8 LD cycle. C) 6/8 Brn3bzDTA; MKO animals exhibit lengthened circadian period in a 6-hr advanced and delayed 16:8 LD cycle. D) Brn3bzDTA; MKO animals do not lengthen their period in LL following 12:12 LD cycle. Red lines indicate main differences in behavior observed.

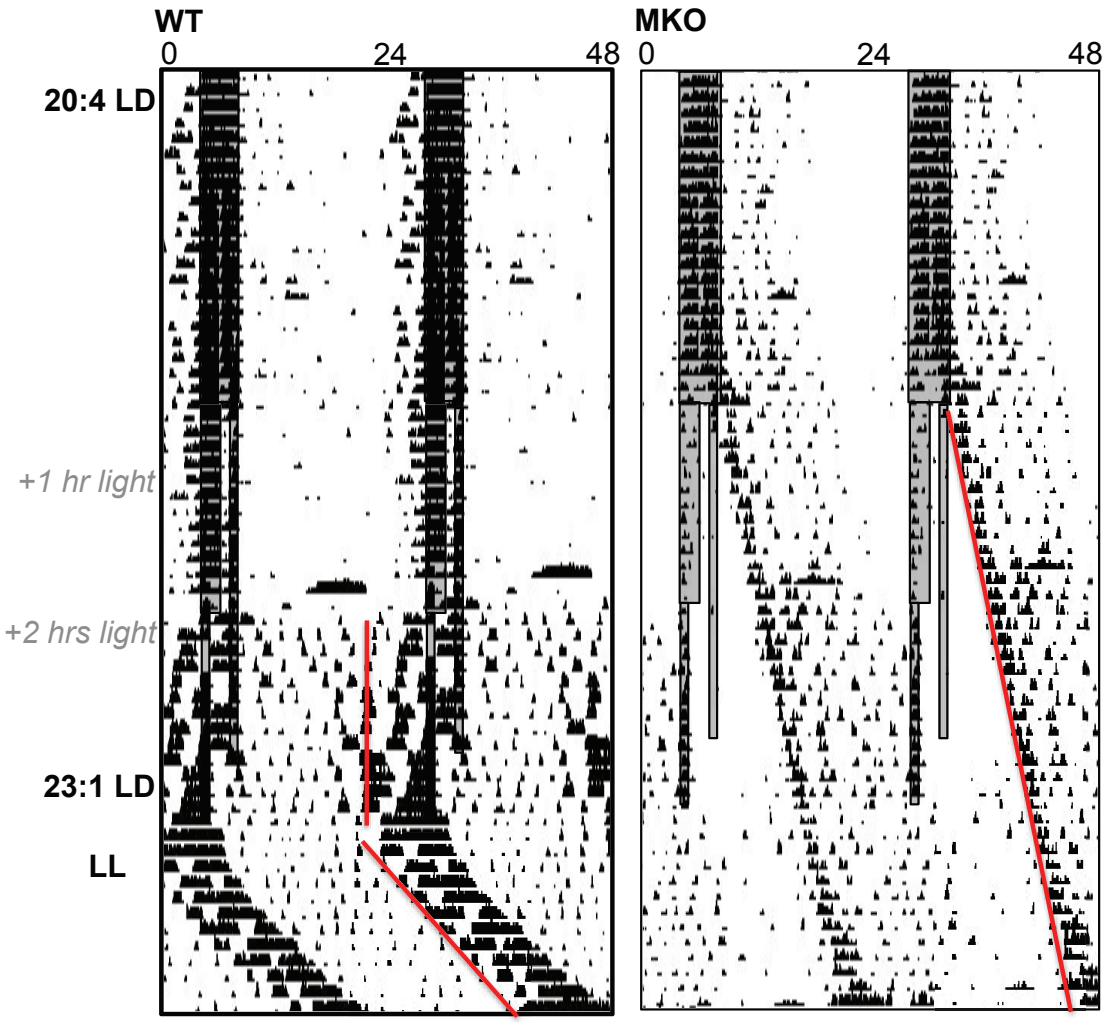


FIGURE 21: Period lengthening occurs in a light disrupted 20:4 LD cycle in a melanopsin independent manner.
Red lines indicate main differences in behavior observed.

DISCUSSION AND CONCLUSIONS

Types of environments that cause period lengthening

While period lengthening occurs under a variety of light dark environments, not all environments can induce a lengthened period. In WT animals specifically, I find that the total amount of light and dark periods in a 24-hr day or the length of light exposure each day does not predict whether period lengthening will occur. Instead, I found that it is the even distribution of multiple light and dark period throughout the day that results in period lengthening.

Melanopsin-dependent versus melanopsin-independent period lengthening

Studying the role of melanopsin in period lengthening allowed some interesting ideas about period lengthening to become apparent. Firstly, melanopsin is important for strong period lengthening responses in LL. Secondly, in irregular LD cycles like T6, MKO animals can also lengthen their period, which suggest that period lengthening may be induced for different reasons. Thirdly, in longer days, LD cycles that have been disrupted by light or shifts in the light dark cycle, I found that MKO, but not WT animals, exhibit period lengthening. This kind of period lengthening may be a response to an environment in which entrainment has become difficult.

Recovery from a lengthened period

The SCN of a nocturnal animal has an intrinsic rhythm of 23.5 hrs in the absence of light input. The external light environment can have the following effects on the clock: entrainment (24 hrs), period lengthening (>24 hrs), or arrhythmicity. My work has found the transition from one type of circadian period to the next is not a trivial matter and relies on the presence of melanopsin in ipRGCs. Melanopsin may act as a "buffer" for sudden and unexpected changes in the LD environment. Further studies are required to understand exactly how melanopsin functions in ipRGCs to drive recovery from aberrant light-dark environments.

So what is period lengthening?

These studies demonstrate period lengthening is a response that occurs in certain disruptive LD environments. Period lengthening may occur because of difficulty entraining to a LD cycle in a melanopsin-dependent or -independent manner. Why does period lengthening occur in these environments rather than arrhythmicity? Arrhythmia is very harsh on the body as each circadian clock is out of synch with the others; this lack of synchronous response in turn severely affects many functions. Period lengthening may be an alternate circadian program that occurs in disruptive light environments in an attempt to maintain a circadian rhythm. Though not entrained with a 24-hr period, it is possible that peripheral clocks may still be able to synchronize to the SCN, although

not the LD environment, and carry out some normal functions. Why period lengthening occurs remains an open and intriguing area for future study.

Chapter 4: Multiple retinal circuits drive circadian photoentrainment in a melanopsin and day length dependent manner

INTRODUCTION

Circadian photoentrainment is the physiological alignment of the circadian biological clock to the earth's day-night cycle. The importance of this alignment is best appreciated when one travels across different time zones and experiences the symptoms associated with "jet lag." In mammals, the retina is the only light sensitive organ that receives light information for circadian photoentrainment. The retina contains three types of photoreceptors; these include the classical photoreceptors, rods and cones, and the intrinsically photosensitive retinal ganglion cells (ipRGCs), which is a unique population of photosensitive retinal ganglion cells (RGCs) that express the photopigment melanopsin (Hattar *et al.,* 2003). At least five distinct subtypes of ipRGCs are found in the mouse retina (M1-M5). Together, they comprise ~4-5% of the total RGC population (Ecker *et al.,* 2010). Recent studies indicate ipRGCs are necessary for circadian photoentrainment (Guler *et al.,* 2008). A specific subpopulation of M1 ipRGCs that does not express the transcription factor Brn3b, innervates the suprachiasmatic nucleus (SCN), to drive circadian photoentrainment at high light intensities (Chen *et al.,* 2011). Because ipRGCs project not only to SCN and but also other brain regions, it is necessary to determine how rods/cones and melanopsin phototransduction pathways contribute to driving circadian photoentrainment through SCN and non-SCN projecting ipRGCs.

Several hypotheses attempt to explain how the circadian period is adjusted to the 24-hour light-dark environment to achieve circadian photoentrainment. The most cited and accepted idea is that light at dawn and dusk adjusts the period of the circadian oscillator to match the 24-hour period (Pittendrigh 1981). This idea is based on the observation that animals maintained under constant dark conditions show advances, delays or no shift in the phase of the circadian oscillator in response to short pulses of light, in what is known as the phase response curve (Minors *et al.,* 1991; Johnson 1990). In light paradigms known as skeleton photoperiods, when only two discrete light pulses are separated by intervening dark, animals photoentrain to the imposed light-dark environments (Schwartz *et al.,* 1996). However, seasonal changes in day length, which affect reproduction in animals, provides evidence for the necessity of measuring light for extended periods as opposed to only discrete light pulses (Bittman and Karsch 1984). In addition, continuous light at night, as long as 6 hours, inhibits the activity of nocturnal animals or melatonin levels in humans (Lewy *et al.,* 1980). This inhibition of activity by light is known as masking, since light can directly mask the circadian regulated increase of activity or melatonin at night. The majority of studies on circadian photoentrainment were carried out before the discovery of melanopsin and ipRGCs. Therefore, the role of ipRGC-mediated light input for circadian photoentrainment remains an open area of study.

Because they are ganglion cells, ipRGCs are not only intrinsically sensitive to light due to melanopsin-mediated photoreception, but also receive light input from rods and cones through the retinal circuitry (Schmidt *et al.,* 2011; Wong 2012). *In vitro* studies show that while both extrinsic and intrinsic inputs are capable of driving ipRGC firing similarly, phase delays in vivo are attenuated in the absence of the intrinsic light response (Wong 2012; Panda *et al.,* 2002). Despite deficits in phase delays, melanopsin knockout animals still show normal circadian photoentrainment to a 24-hour light dark cycles, which indicates the phase response curve is not a reliable predictor of circadian photoentrainment (Panda *et al.,* 2002; Ruby *et al.,* 2002). The most notable deficits in melanopsin knockout animals are obtained in situations where prolonged light measurements are required, such as masking for 3 hours in nocturnal rodents, melatonin inhibition for 6 hours in humans or lengthening of the circadian period under constant light conditions (Hattar *et al.,* 2003; Lewy *et al.,* 1980; Altimus *et al.,* 2010; Decoursey 1986). These deficits in melanopsin knockout animals raise the intriguing possibility that the contribution of melanopsin phototransduction to circadian light responses is day length dependent. In this study, we investigate the importance of rods/cones and melanopsin based phototransduction to a variety of light-dark paradigms and correlate the effects of phase shifting and masking to circadian photoentrainment.

METHODS

Mice

All mice were of a mixed background (BL/6;129SvJ). Male animals that were used in the behavioral analyses were aged between 3 and 12 months. Animals were housed and treated in accordance with NIH and IACUC guidelines, and used protocols approved by the Johns Hopkins University Animal Care and Use Committees.

Types of mouse models

During this chapter, "Control" refers to WT mice. Mice that lack the production of functional melanopsin protein in their ipRGCs are refered to a melanopsin knockout ("MKO"). These MKO animals were previously published *Opn4*$^{-/-}$. "Brn3bzDTA" refer to mice in which the Brn3b-positive ipRGCs are ablated, and thus only the Brn3b-negative ipRGCs remain. There are two flavors of this mouse: the "Brn3bzDTA; Cre/+" (previously published as *Opn4*$^{Cre/+}$*; Brn3b*$^{Z\text{-}dta/+}$*)* in which one functional copy of the melanopsin gene remains to produce melanopsin protein, and the "Brn3bzDTA; MKO" (previously published as *Opn4*$^{Cre/+}$*; Brn3b*$^{Z\text{-}dta/+}$) in which there is no melanopsin protein (thus an "MKO").

Wheel-running activity

Mice were placed in cages with a 4.5-inch running wheel, and their activity was monitored with VitalView software (MiniMitter). Analyses of wheel running activity were calculated with ClockLab (Actimetrics). Unless noted otherwise, we used 500 lux light intensity (moderately bright-office lighting). Mice were initially placed under 12:12 LD or 16:8 LD cycle for 2 weeks before any jet lag experiments. Phase-shifting experiments were carried out on the seventh day of constant darkness when each animal was exposed to a 15 min light pulse at CT (circadian time) 14 or CT 22 (500 lux). The light intensity for all masking experiments was also 500 lux.

Transition between light cycles in the jet lag paradigm

6-hr phase delay

To account for the difference in time experienced when delaying the LD cycle during the jet lag paradigm, the last night of the previous LD cycle is lengthened (from 12 hrs to 18 hrs in 12:12 LD and from 8 hrs to 14 hrs in 16:8 LD). Therefore, during the delayed 12:12 LD cycle, there is an 18-hour night that precedes the first 12-hour day in the newly delayed 12:12 LD cycle. During the delayed 16:8 LD cycle, there is a 14-hour night that precedes the first 16-hour day in the newly delayed 16:8 LD cycle.

6-hr phase advance

To account for the difference in time experienced when advancing the LD cycle during the jet lag paradigm, the last day of the previous LD cycle is shortened (from 12 hrs to 6 hrs in 12:12 LD and from 16 hrs to 10 hrs in 16:8 LD). Therefore, during the advanced 12:12 LD cycle, there is a 6-hour day that precedes the first 12-hour night in the newly advanced 12:12 LD cycle. During the advanced 16:8 LD cycle, there is a 10-hour day that precedes the first 8-hour night in the newly advanced 16:8 LD cycle.

Statistical analysis

All statistical analysis was performed using GraphPad Prism. Statistical analysis for each graph is specified in the figure legend.

RESULTS

Day length affects the contribution of melanopsin in driving circadian photoentrainment.

Previous studies have shown that animals lacking the melanopsin protein in ipRGCs (referred to as "MKO"-melanopsin knockout), are able to photoentrain to a 12hr:12hr light-dark (12:12 LD) cycle similar to wildtype (control) animals (Panda *et al.,* 2002; Figure 22 C and D). I investigated the role of melanopsin in photoentrainment under different day lengths and observed the following findings: In 8:16 LD, MKO animals exhibited an advanced angle of entrainment with relation to the LD environment as compared to WT animals (Figure 22 A and B). This behavior was also observed in 16:8 LD (Figure 22 E and F). Similar to the photoentrainment of WT and MKO animals in 12:12 LD, both WT and MKO animals entrained to the 20:4 LD cycle (Figure 22 G and H). WT animals maintained an alignment with the LD environment in 23:1 LD although they exhibited a large advanced phase angle of alignment (Figure 22 I and J). MKO animals exhibited a small amount of activity during the dark but displayed a lengthened period simultaneously in 23:1 LD (Figure 22 I and J). Together, these findings indicate that circadian photoentrainment is variable depending on the LD environment and contribution of melanopsin.

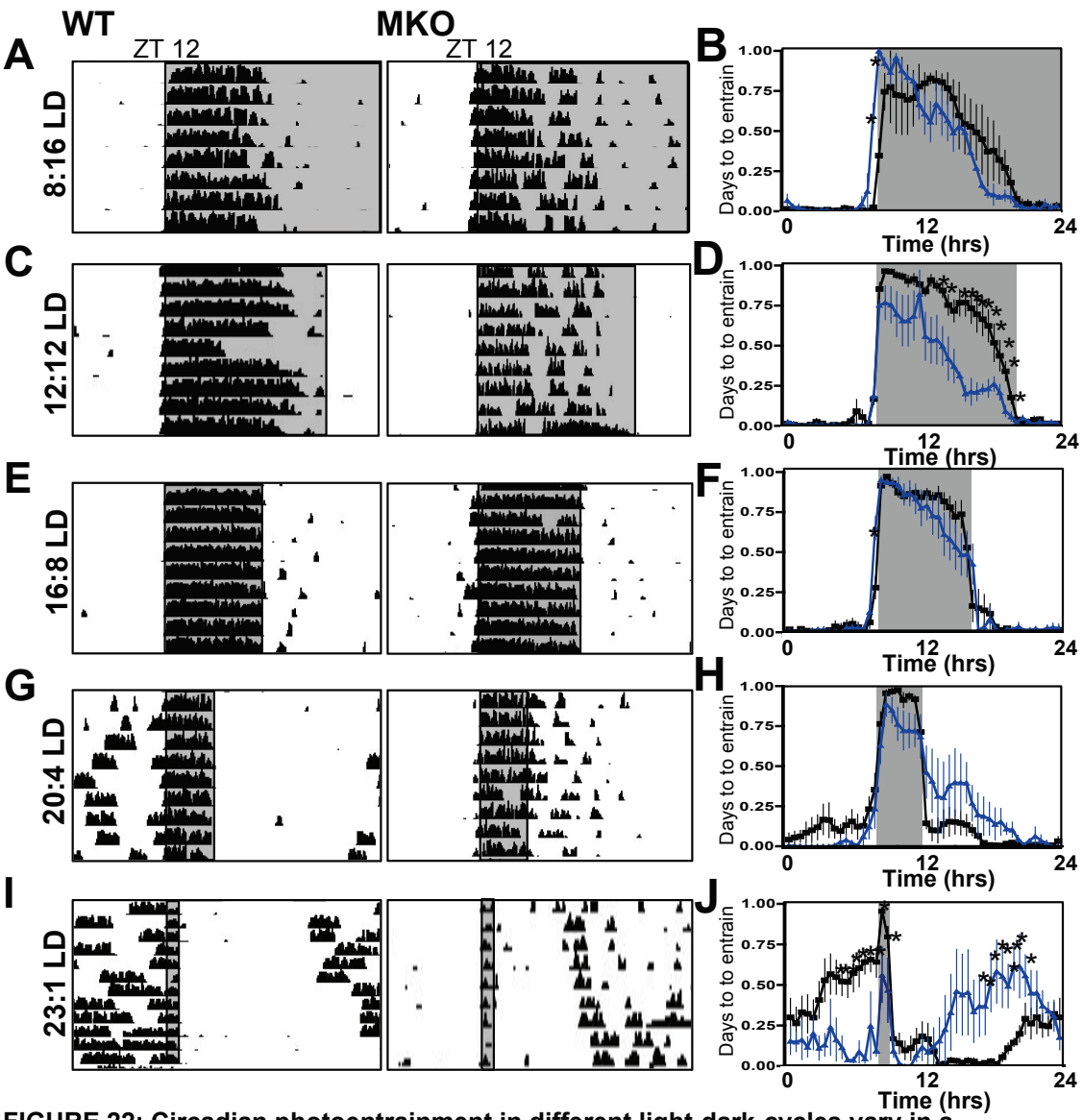


FIGURE 22: Circadian photoentrainment in different light-dark-cycles vary in a melanopsin-dependent manner.

A) Photoentrainment in 8:16 LD cycle. B) Daily average distribution of activity in WT and MKO animals in 8:16 LD. Data graphed in 1-hr bins. C) Photoentrainment in 12:12 LD cycle. D) Daily average distribution of activity in WT and MKO animals in 12:12 LD. Data graphed in 1-hr bins. E) Photoentrainment in 16:8 LD cycle. F) Daily average distribution of activity in WT and MKO animals in 16:8 LD. Data graphed in 1-hr bins. G) Photoentrainment in 20:4 LD cycle. H) Daily average distribution of activity in WT and MKO animals in 20:4 LD. Data graphed in 1-hr bins. I) Photoentrainment in 23:1 LD cycle. J) Daily average distribution of activity in WT and MKO animals in 23:1 LD. Data graphed in 1-hr bins. Statistical analysis: Two-way ANOVA. P-value: *P<0.05.

Re-entrainment in a jet lag paradigm is melanopsin-dependent under specific day lengths.

To further study circadian photoentrainment, I subjected mice to 6-hour changes in the onset of the light and dark environment using a jet lag paradigm. Using this paradigm, I shifted the LD cycle 6 hours earlier (phase advance) followed by a 6-hr shift later (phase delay). During the 16:8 LD, both control and MKO animals photoentrain to the 6-hour phase advance in the LD cycle. MKO animals take significantly longer to photoentrain to the 6-hour phase delay and the subsequent 6-hour phase advance (Figure 23 A and D). During the 12:12 LD cycle, control and MKO animals photoentrain to the 6-hour phase advance and the 6-hour phase delay within 5 days (Figure 23 B and E). During the 8:16 LD cycle, control and MKO animals photoentrain to the 6-hour phase delay within and the 6-hour phase delay within 3-5 days, which is similar to what I observed in 12:12 LD (Figure 23 C and F). Both control and MKO animals took longer to re-entrain the 6-hr phase advance of the 8:16 LD cycle (Figure 23 C and F). However, control animals but not MKO animals, show a significantly slower rate of re-entrainment (Figure 23 C and F). I found that control animals re-entrain more quickly to 6-hour phase advance in shorter nights as compared to longer nights (Figure 23 G). This effect of night length does not occur in the 6-hour phase delayed LD cycles. In contrast, MKO animals took significantly longer to re-entrain to both the 6-hour phase advance and the 6-hour phase delay

under the 16:8 LD cycle, with the larger latency in re-entraining observed during the 6-hour phase delay (Figure 23 H). I did not notice any day length dependent trends in MKO re-entrainment.

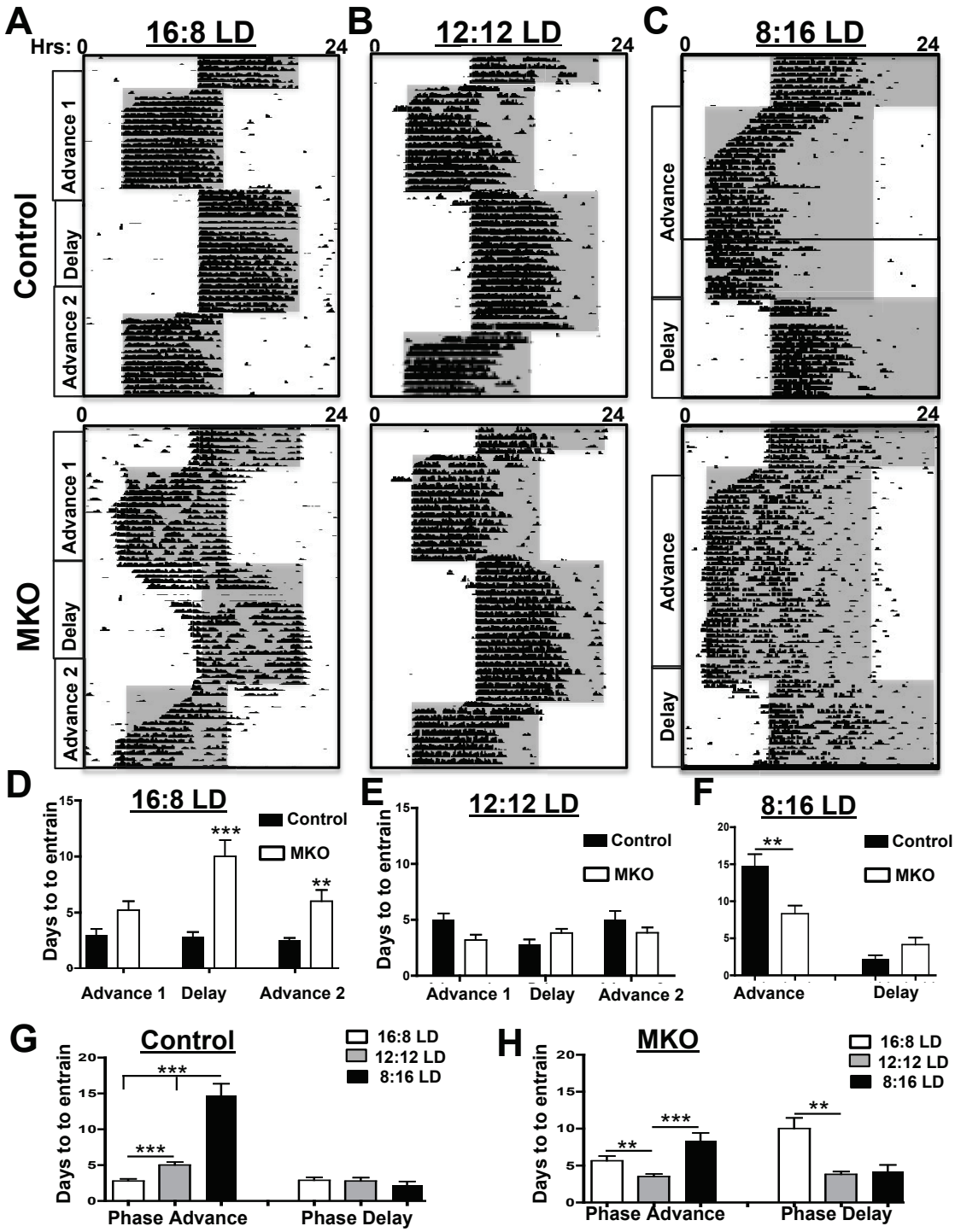

A
16:8 LD
Hrs: 0
24
B
12:12 LD
C
8:16 LD
Control
MKO
Advance 1
Delay
Advance 2
Advance
D
16:8 LD
E
12:12 LD
F
8:16 LD
Days to entrain
Control
MKO
***
**
Advance 1
Delay
Advance 2
Advance
G
Control
H
MKO
16:8 LD
12:12 LD
8:16 LD
Phase Advance
Phase Delay

FIGURE 23: Circadian photoentrainment to the jet lag paradigm during different LD cycles varies in a melanopsin–dependent and day length dependent manner.
A) Re-entrainment in 8:16 LD cycle in control and MKO animals. B) Re-entrainment in 12:12 LD cycle in control and MKO animals. C) Re-entrainment in 16:8 LD cycle in control and MKO animals. D) Quantification of number of days to re-entrain to the 6-hr phase advance 1, 6-hr phase delay, and 6-hr phase advance 2 during the 16:8 LD cycle. E) Quantification of number of days to re-entrain to the 6-hr phase advance 1, 6-hr phase delay, and 6-hr phase advance 2 during the 12:12 LD cycle. F) Quantification of number of days to re-entrain to the 6-hr phase advance 1, 6-hr phase delay, and 6-hr phase advance 2 during the 8:16 LD cycle. G) Average number of days to re-entrain to 6-hr phase advance and 6-hr phase delay in Control animals in 16:8 LD, 12:12 LD and 8:16 LD. H) Average number of days to re-entrain to 6-hr phase advance and 6-hr phase delay in MKO animals in 16:8 LD, 12:12 LD and 8:16 LD. Statistical analysis: two-tailed student's t-test. P-values: **P<0.01, ***P<0.001.

Melanopsin is important for the acute suppression of activity by light in light-dark cycle of specific day lengths.

As they re-entrain to the 6-hour delayed 16:8 LD cycle, MKO animals do not suppress their wheel running activity, that is, they do not mask), whereas they exhibit a strong masking response under 12:12 LD cycle. I measured the amount of activity in the first day after the LD cycle shift in the 16:8 LD and 12:12 LD cycles, as well as the 8:16 LD cycle, to quantify the masking deficits under different light treatments. I found that control animals maintained under 16:8 LD showed some masking deficits when exposed to 10 hours of light, while all MKO animals exhibited masking deficits (Figure 24 A-D). During the 12:12 LD cycle, however, and following 6-hours of light exposure, both control and MKO animals exhibit similar masking responses to light (Figure 24 E and 24 F). Similar findings are observed in 8:16 LD as compared with 12:12 LD (Figure 24 G and H).

Using the more traditional masking paradigm, in which a 3-hour light pulse is administered to mice during the early part of the night, I tested whether 2-hours of dark adaptation was sufficient for the rods/cones to dark adapt and drive masking in MKO animals. I found that MKO animals mask to 3-hours of light following 12-hour prior light exposure and 2-hours dark adaptation (Figure 25 C and D). MKO animals, however, could not mask to 3-hours of light following 16-hour

(in 16:8 LD) or 8-hour (in 8:16 LD) prior light exposure, even with 2-hours dark adaptation (Figure 25 A, B, E and F).

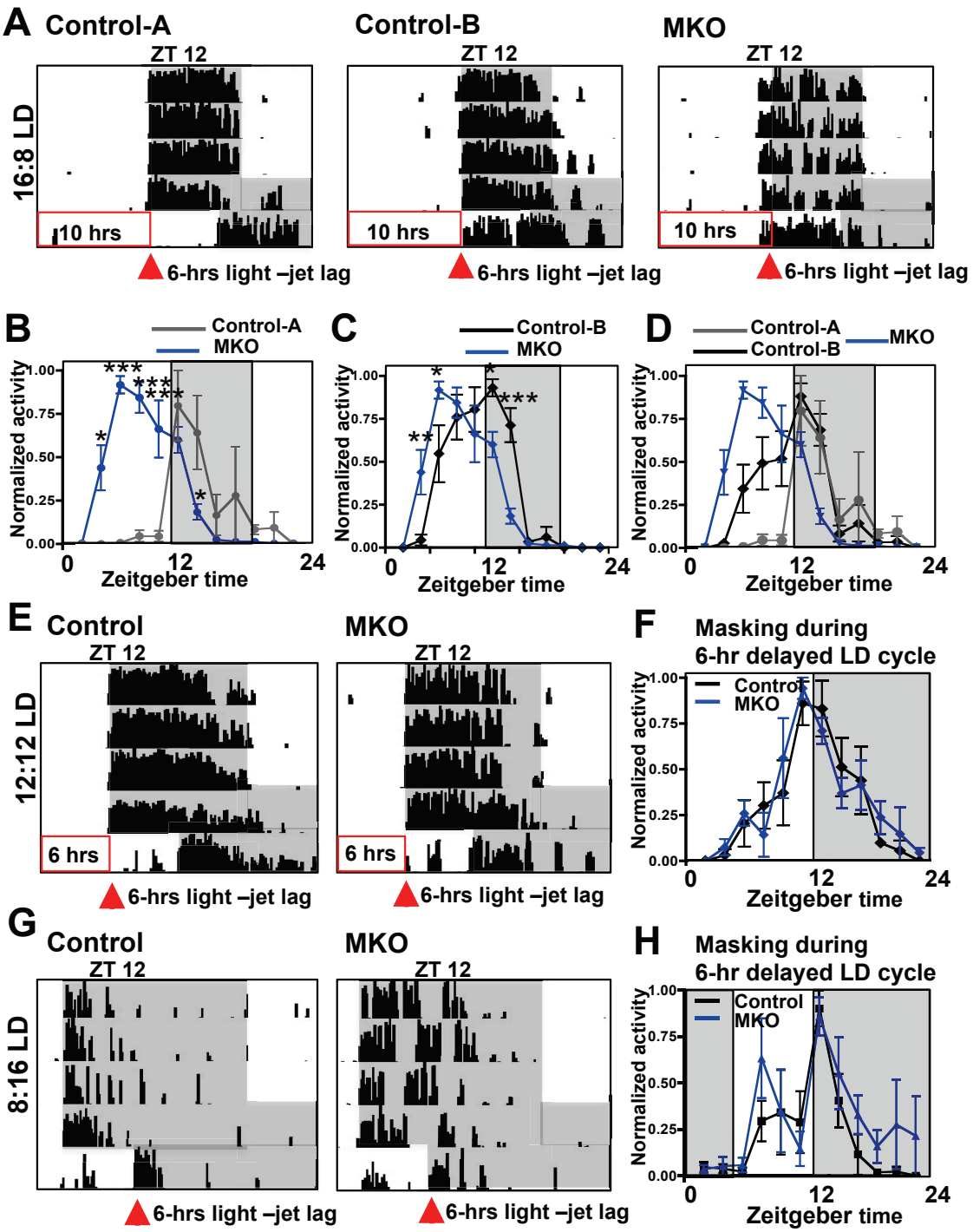

A
Control-A
Control-B
MKO
ZT 12
16:8 LD
10 hrs
6-hrs light –jet lag
B
C
D
Control-A
Control-B
MKO
Normalized activity
Zeitgeber time
E
Control
MKO
12:12 LD
6 hrs
F
Masking during
6-hr delayed LD cycle
Control
G
8:16 LD
H

FIGURE 24: Light history influences negative masking during the jet lag paradigm in a day length-dependent manner.

A) Masking on the first day in the 6-hr delayed 16:8 LD cycle in Control and MKO animals. Red box indicates the 6 hours of light exposure before the time of masking (indicated by red arrow). Two phenotypes observed for Controls: Control-A- n=3/7, Control-B- n=4/7. B) Quantification of masking on the first day of the 6-hr delayed LD cycle for Control-A and MKO. C) Quantification of masking on the first day of the 6-hr delayed LD cycle for Control-B and MKO. D) Quantification of masking on the first day of the 6-hr delayed LD cycle for Control-A, Control-B and MKO. E) Masking on the first day in the 6-hr delayed 12:12 LD cycle in Control and MKO animals. Red box indicates the 6 hours of light exposure before the time of masking (indicated by red arrow). F) Quantification of masking on the first day of the 6-hr delayed LD cycle. ZT 12 refers to the onset of darkness in the 6-hr delayed LD cycle. G) Masking on the first day in the 6-hr delayed 8:16 LD cycle in Control and MKO animals. Red box indicates the 6 hours of light exposure before the time of masking (indicated by red arrow). H) Quantification of masking on the first day of the 6-hr delayed LD cycle. ZT 12 refers to the onset of darkness in the 6-hr delayed LD cycle. Statistical analysis: Two-way ANOVA. P-values: *P<0.05, **P<0.01, ***P<0.001.

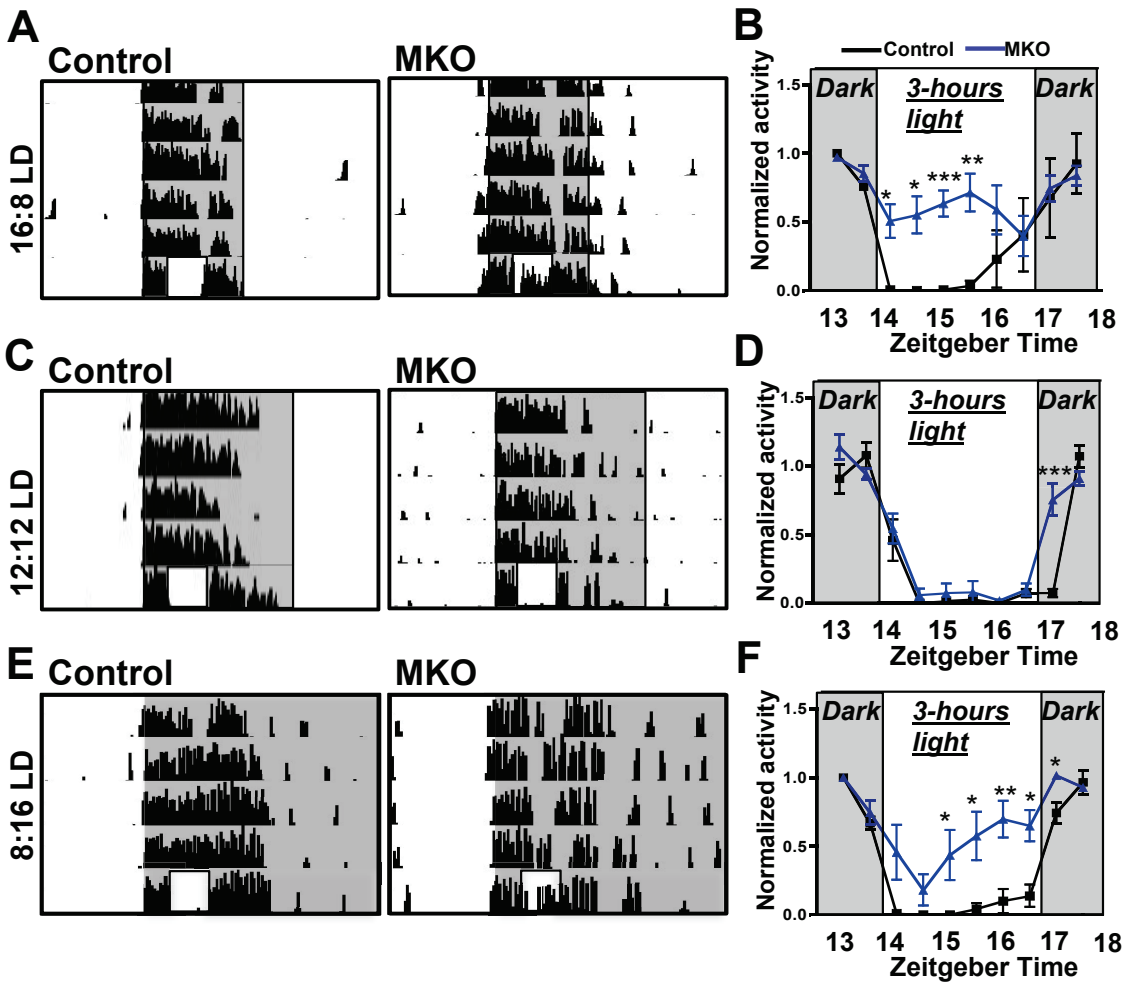


FIGURE 25: Light history influences negative masking during the early night in a day length-dependent manner.

A) Masking to 3-hr light exposure at ZT 14 - ZT 17 during the 16:8 LD cycle in Control and MKO animals. B) Quantification of activity before, during and after 3-hr light exposure at ZT 14 - ZT 17. Activity graphed in 30-minute bins and normalized to activity one-hour prior to light pulse for each animal. C) Masking to 3-hr light exposure at ZT 14 - ZT 17 during the 12:12 LD cycle in Control and MKO animals. D) Quantification of activity before, during and after 3-hr light exposure at ZT 14 - ZT 17. Activity graphed in 30-minute bins and normalized to activity one-hour prior to light pulse for each animal. E) Masking to 3-hr light exposure at ZT 14 - ZT 17 during the 8:16 LD cycle in Control and MKO animals. F) Quantification of activity before, during and after 3-hr light exposure at ZT 14 - ZT 17. Activity graphed in 30-minute bins and normalized to activity one-hour prior to light pulse for each animal. Statistical analysis: Two-way ANOVA. P-values: *P<0.05, **P<0.01, ***P<0.001.

Deficits in both masking and circadian phase shifting correlate to the deficits observed in circadian photoentrainment during the jet lag paradigm.

MKO animals show deficits in circadian phase delays (Panda *et al.,* 2002; Figure 26 A and B). Consistent with the deficits in phase delays, MKO animals show weaker induction of c-fos in the SCN during the early night (See chapter 2, Figures 1-3), but not late night (See chapter 2, Figure 1-3). MKO animals, however, exhibit normal circadian photoentrainment under 12:12 LD (Figure 22) despite deficits in phase shifting, which suggest circadian photoentrainment is not a direct reflection of the phase response curve. Phase advance of the clock to a 15 mins light pulse at CT 22 induces phase shifts of similar magnitudes in both WT and MKO animals (Figure 26 C and D).

Studying phase shifting in constant darkness is not likely to provide the most accurate prediction of circadian phase responses in a light-dark environment. To study phase delays in the context of the light dark environment, I kept mice in either 16:8 LD, 12:12 LD, or 8:16 LD and exposed them to 3-hours of light 2-hours after dark onset. Under 16:8 LD, MKO animals do not phase delay or mask to the 3-hour light treatment (Figure 26 E and H) and they do experience major deficits in re-entrainment. Control animals, by contrast, maintain masking, but lack phase delays, and still exhibit normal re-entrainment. In 12:12 LD I

found that both control and MKO animals mask in 12:12 LD, but only control animals show a phase delay (Figure 26 F and H). In 8:16 LD, similar to what I observed in 12:12 LD, controls exhibited masking and phase shifting, while MKO animals exhibited deficits in both phase shifting and masking (Figure 26 G and H). The absence of phase delays in MKO animals did not hinder their ability to re-entrain to the 6-hr delayed 12:12 LD cycle or 8:16 LD, which implicates a role for masking and day length measuring in photoentrainment.

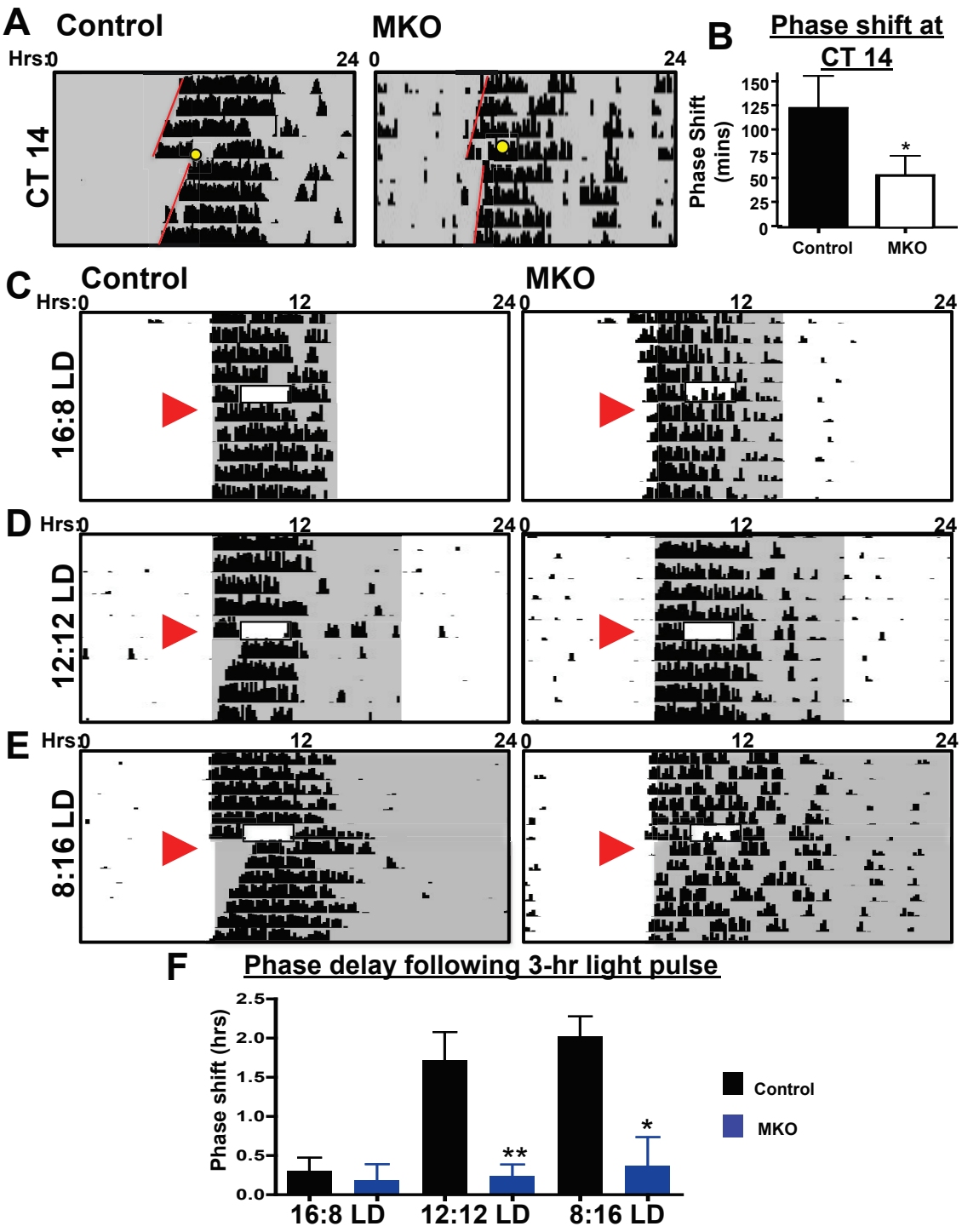

A
Control
MKO
Hrs:
0
24
CT 14
B
Phase shift at CT 14
Phase Shift (mins)
150
125
100
75
50
25
0
*
Control
MKO
C
Control
MKO
16:8 LD
12
D
12:12 LD
E
8:16 LD
F
Phase delay following 3-hr light pulse
Phase shift (hrs)
2.5
2.0
1.5
1.0
0.5
0.0
**
*
16:8 LD
12:12 LD
8:16 LD
Control
MKO

FIGURE 26: Melanopsin is important for both phase shifting and masking in 16:8 LD, which correlates with deficits observed in MKO animals during the 6-hr delayed 16:8 LD cycle.
A) Actogram depicting phase delay to 15 minutes of light at circadian time (CT) 14 in control and MKO animals. B) Quantification of phase delay. C) Phase shift induced by 3-hr light exposure at ZT 14 - ZT 17 during the 16:8 LD cycle in control and MKO animals. First day of shift indicated with red arrowhead. D) Phase shift induced by 3-hr light exposure at ZT 14 - ZT 17 during the 12:12 LD cycle in control and MKO animals. First day of shift indicated with red arrowhead. E) Phase shift induced by 3-hr light exposure at ZT 14 - ZT 17 during the 8:16 LD cycle in control and MKO animals. First day of shift indicated with red arrowhead. F) Quantification of phase shift induced by 3-hr light exposure at ZT 14 – ZT 17 during the 16:8, 12:12, and 8:16 LD cycles in control and MKO animals. Statistical analyses: two-tailed student's t-test. P-values: *P<0.05, **P<0.01.

Melanopsin is necessary in SCN-projecting Brn3b-negative ipRGCs for normal circadian phase alignment in 12:12 LD.

No more than 200 Brn3b-negative M1 ipRGCs that innervate the SCN are sufficient for circadian photoentrainment in the presence of the melanopsin protein (Altimus *et al.,* 2010). To determine if these cells are sufficient for signaling rod/cone input to the SCN for circadian photoentrainment, masking and phase shifting in the 12:12 LD cycle, I generated animals in which the 200 M1 Brn3b-negative ipRGCs that also lack melanopsin; the "Brn3bzDTA; MKO" animals. IpRGCs in these animals signal light exclusively through rod/cone input. "Brn3bzDTA; Cre/+" animals were used to control to the presence of melanopsin in the Brn3b-negative ipRGCs (Chen *et al.,* 2011). In the 12:12 LD cycle, melanopsin expressing animals closely resemble control animals in all circadian behaviors tested (Chen *et al.,* 2011) but those animals with no melanopsin expression display significant deficits in entrainment, as observed by their advanced phase angle and unstable activity onset (Figure 27 A-C).

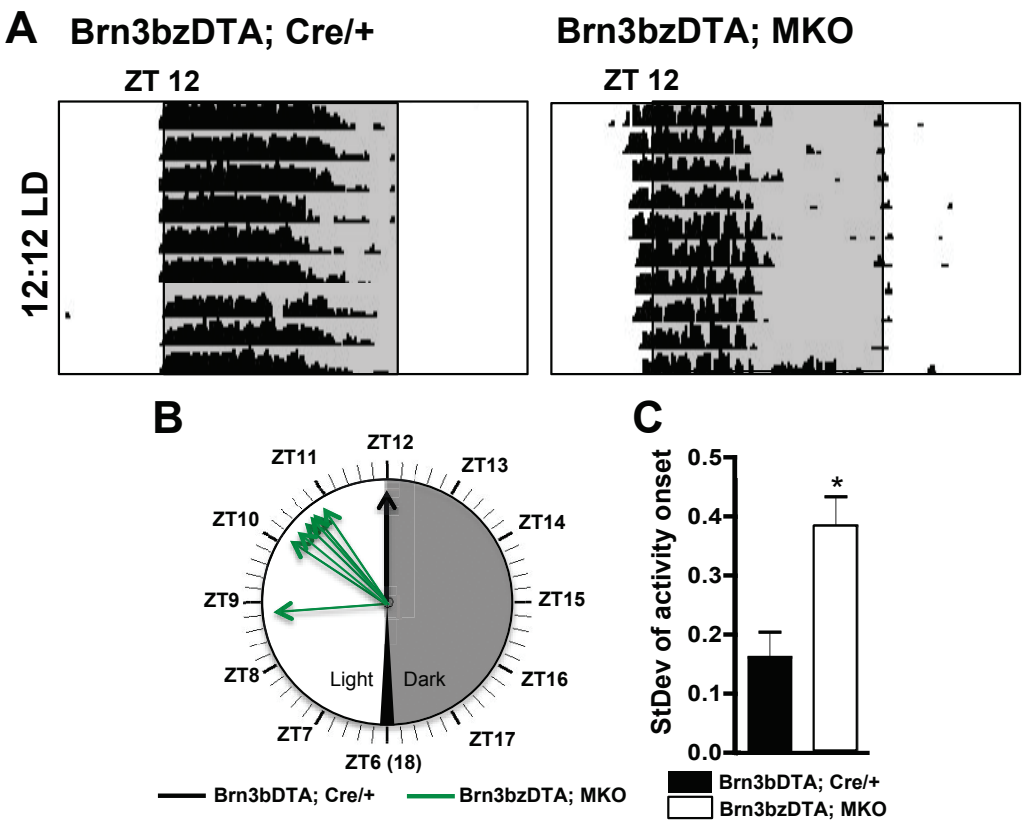


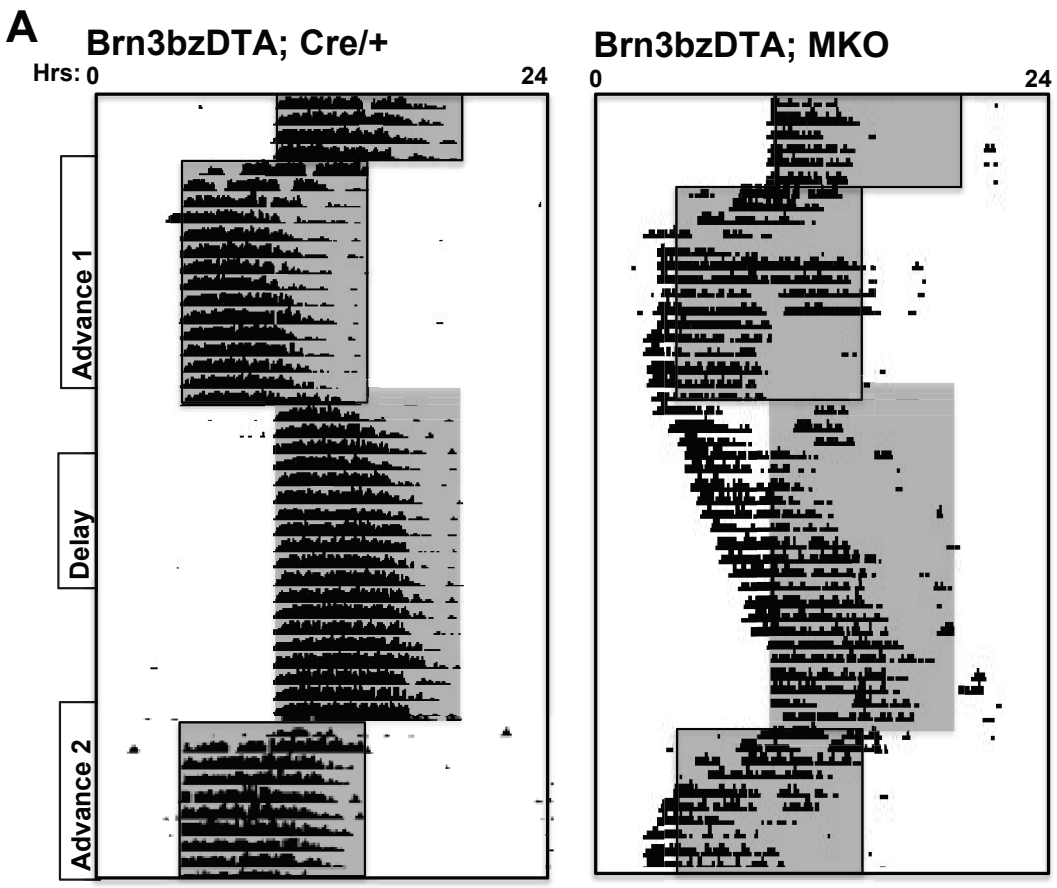
FIGURE 27: Melanopsin is necessary in Brn3b-negative ipRGCs to drive precise phase alignment in the 12:12 LD cycle.
A) Photoentrainment to a 12:12 LD cycle in Brn3bzDTA; Cre/+ and Brn3bzDTA; MKO animals. B) Phase angle of alignment for Brn3bzDTA; Cre/+ and Brn3bzDTA; MKO animals in 12:12 LD cycle. Average alignment is shown to ZT12 for Brn3bzDTA; Cre/ + animals. Each green arrow represents activity onset for a single Brn3bzDTA; MKO mouse. C) Stability of activity onset in 12:12 LD cycle for Brn3bzDTA; Cre/+ and Brn3bzDTA; MKO animals; measured by the standard deviation in activity onset over 10 days. Statistical analyses: two-tailed student's t-test. P-values: *P<0.05.

Melanopsin is necessary in Brn3b-negative ipRGCs for normal photoentrainment during the jet lag paradigm in 12:12 LD.

In figure 23, I reported that melanopsin is necessary for circadian photoentrainment in the jet lag paradigm in a day length dependent manner. The MKO animals used in figure 23 had no melanopsin in all ipRGCs, including those ipRGCs that project to the SCN and non-SCN brain regions. It is therefore possible that non-SCN brain regions, in addition to the SCN, may use the rod/cone input through ipRGCs to drive normal photoentrainment in some day lengths but not others. To further investigate the contribution of rods/cones to circadian photoentrainment through the Brn3b-negative ipRGCs, I subjected mice to the 6-hour jet lag paradigm in the 12:12 LD cycle. I observed that Brn3bzDTA; MKO animals had normal re-entrainment to the 6-hour phase advance, but took significantly longer to photoentrain to the 6-hour delayed 12:12 LD cycle (Figure 28 A and B). Thus Brn3bzDTA; MKO animals under 12:12 LD cycle have circadian photoentrainment deficits similar to MKO animals under 16:8 LD.

To determine if the deficits observed in the Brn3bzDTA; MKO animals but not the Brn3bzDTA; Cre/+ animals is solely due the presence of melanopsin, I next tested photoentrainment at dim light intensities, below the sensitivity threshold of melanopsin photo-response (Altimus *et al.,* 2010). In a 12:12 LD cycle, I lowered the light intensity to

10 lux and to 1 lux concurrent with a 6-hr phase advance, to determine if re-entrainment was affected in the Brn3bzDTA; Cre/+ animals (Figure 29 A). I found that at both 10 lux and 1 lux, where melanopsin sensitivity to light is drastically reduced, MKO, Brn3bzDTA; Cre/+ and Brn3bzDTA; MKO animals all exhibited a significantly larger advanced phase angle of alignment to the LD cycle (Figure 29 B). However, I found that only Brn3bzDTA; Cre/+ and Brn3bzDTA; MKO took significantly longer to re-entrain to a 6-hr phase advance at 1 lux (Figure 29 C). In addition, during the re-entrainment, both animals showed 20% of their total activity under the light portion of the LD cycle (Figure 29 D).



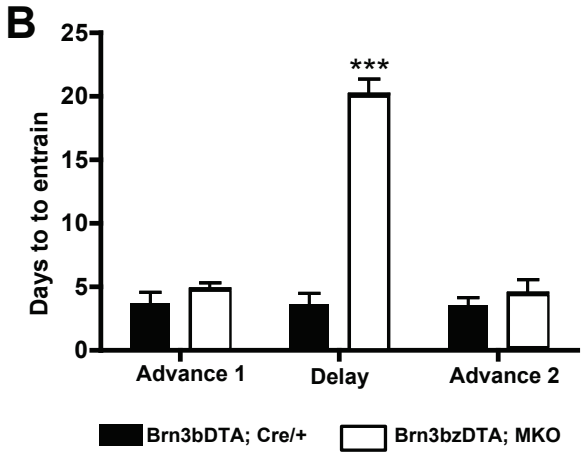


FIGURE 28: Melanopsin is necessary in Brn3b-negative ipRGCs to rapid re-entrainment to 6-hr delayed 12:12 LD cycle at high light intensities.
A) Re-entrainment to 6-hr phase advance, 6-hr phase delay, and 6-hr phase advance in a 12:12 LD for Brn3bzDTA; Cre/+ and Brn3bzDTA; MKO animals at 500 lux. B) Quantification of number of days to re-entrain to the 6-hr phase advances and delay during the 12:12 LD cycle.
Statistical analyses: two-tailed student's t-test. P-value: ***P<0.001.

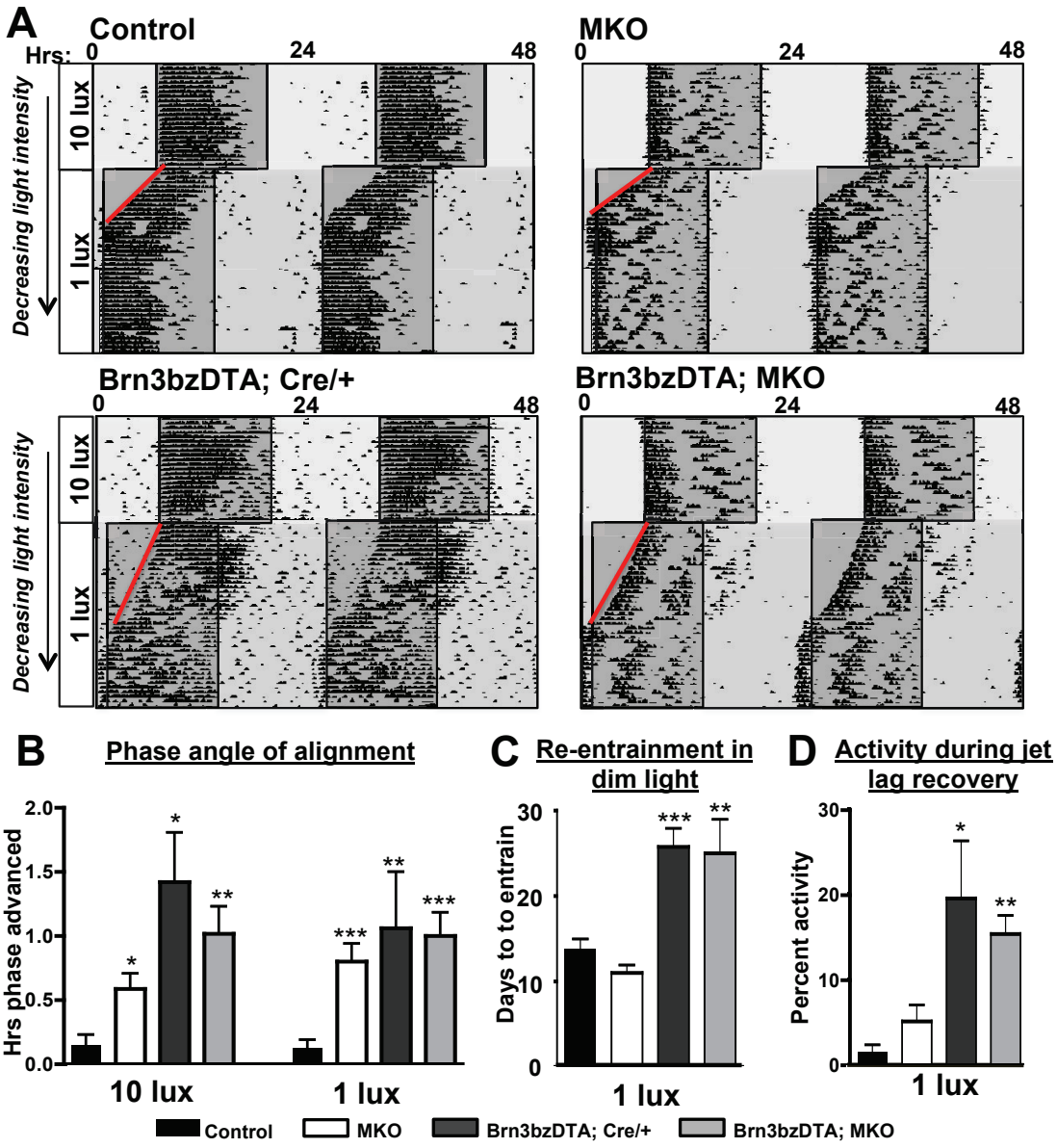


FIGURE 29: Melanopsin is necessary in Brn3b-negative ipRGCs to rapid re-entrainment in dim light intensities during the 12:12 LD cycle.

A) Re-entrainment to a 6-hr phase advance from 10 lux to 1 lux. B) Phase angle of alignment while photoentrained in 10 lux and 1 lux. C) Number of days to re-entrain to t6-hr phase advance at 10 lux. D) Average percent of daily activity under the light while re-entraining to 6-hr phase advance at 10 lux. Statistical analyses: two-tailed student's t-test. P-values: *P<0.05, **P<0.01, ***P<0.001.

Phase shifting to 15 minutes light pulse during the darkness in Brn3bzDTA; MKO animals is similar to MKO animals.

Consistent with the deficits observed in phase delays and c-fos induction in MKO animals, Brn3bzDTA; MKO animals also show small phase shift in response to light during the early night (CT 14) (Figure 30 A and B). This deficit in phase shifting is consistent with the deficits in re-entrainment to the 6-hr delayed 12:12 LD cycle. In addition, similar to the normal re-entrainment observed in the Brn3bzDTA; MKO animals during the 6-hr phase advanced 12:12 LD cycle, I found that phase advancing to a 15-mins light pulse administered during the late night (CT 22) was similar to Brn3bzDTA; Cre/+ (Figure 30 C and D).

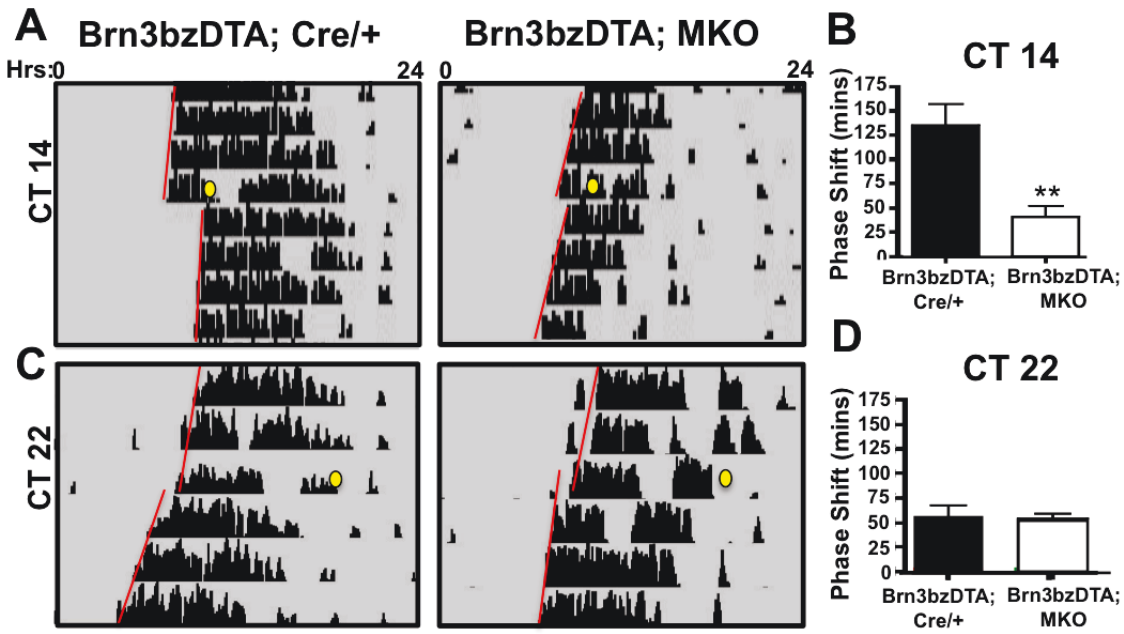


FIGURE 30: The presence of melanopsin is necessary in Brn3b-negative ipRGCs for WT-like phase shifting to 15 minutes of light during the darkness.
A) 15-minutes light exposure at CT 14 during constant darkness in Brn3bzDTA; Cre/+ and Brn3bzDTA; MKO animals. B) Quantification of phase shift at CT 14. C) 15-minutes light exposure at CT 22 during constant darkness in Brn3bzDTA; Cre/+ and Brn3bzDTA; MKO animals. D) Quantification of phase shift at CT 22. Statistical analyses: two-tailed student's t-test. P-values: *P<0.05, **P<0.01.

Phase shifting occurs in multiple LD environments and depends on how much the retinal circuit is intact.

In keeping with my findings that light can induce phase delays in non-DD environments, I further studied how light can induce phase delays. While maintained in a 12:12 LD cycle, I administered 3 hours of light at ZT 14. On the following day, when a phase shift should result, I also administered a 6-hr phase advance and reduced the light intensity to 10 lux. These changes in the LD environment would not affect the phase shift that should result but affect the recovery from the phase shift inducing 3-hour light pulse.

I found that control animals had a phase shift of an unprecedented 4 hours (Figure 31 A and B). Interestingly, both MKO and Brn3bzDTA; Cre/+ animals had a very similar magnitude of phase shift of 2.5 hours that was much smaller than control animals (Figure 31 A and B). The phase shift of both the control and MKO animals are twice the magnitude of the phase shift I observed while mice were maintained in 12:12 LD (see Figure 26). I also found that Brn3bzDTA; MKO animals had little phase shift in this paradigm (Figure 31A and B).

I also discovered that when placed into LL from 12:12 LD, mice will exhibit a delay in the phase of their activity onset. Again, control animals exhibited the largest phase shift of 5 hours (Figure 31 C and D). Also, I found that MKO and Brn3bzDTA; Cre/+ animals once again exhibited a

phase shift of 2.5 hours that is significantly smaller than control animals. Finally, Brn3bzDTA; MKO animals exhibited very little phase shifting in this paradigm (Figure 31 C and D).

Together, these data suggests that the phase shifting observed in Brn3bzDTA; MKO animals during DD may be due to the hypersensitive nature of rods and cones when maintained in constant darkness for an extended time. However, the phase shift (delay) may be enhanced by the presence of melanopsin. It is also interesting to note what appears to be a dose responsiveness of the co-operative ipRGC and melanopsin system, in that when either the number of ipRGCs or the presence of melanopsin is disrupted, there is an intermediate phenotype.

Masking is again correlated with deficits in circadian photoentrainment in the Brn3bzDTA; MKO animals.

In contrast to MKO animals under 12:12 LD, Brn3bzDTA; MKO animals exhibit deficits in photoentrainment in the jet lag paradigm, suggesting that SCN is not the only brain region involved in mediating rod/cone input to circadian photoentrainment. To determine if the deficits in circadian photoentrainment in Brn3bzDTA; MKO animals are due to masking deficits similar to those observed in MKO animals in 16:8 LD, I analyzed masking under conditions similar to those used in Figure 24. I find that in 12:12 LD, melanopsin is necessary in the SCN–projecting Brn3b-negative ipRGCs for masking on the first day in the 6-hour

delayed LD cycle (Figure 32 A and B). I also find that Brn3bzDTA; MKO animals exhibit masking deficits to 12 hours light (Figure 32 C and D) and 3 hours light (Figure 32 E and F) at night as compared to Brn3bzDTA; Cre/+ animals and MKO animals. This data provides more evidence that masking is important for circadian photoentrainment. In addition, the SCN is sufficient for masking only in the presence of melanopsin.

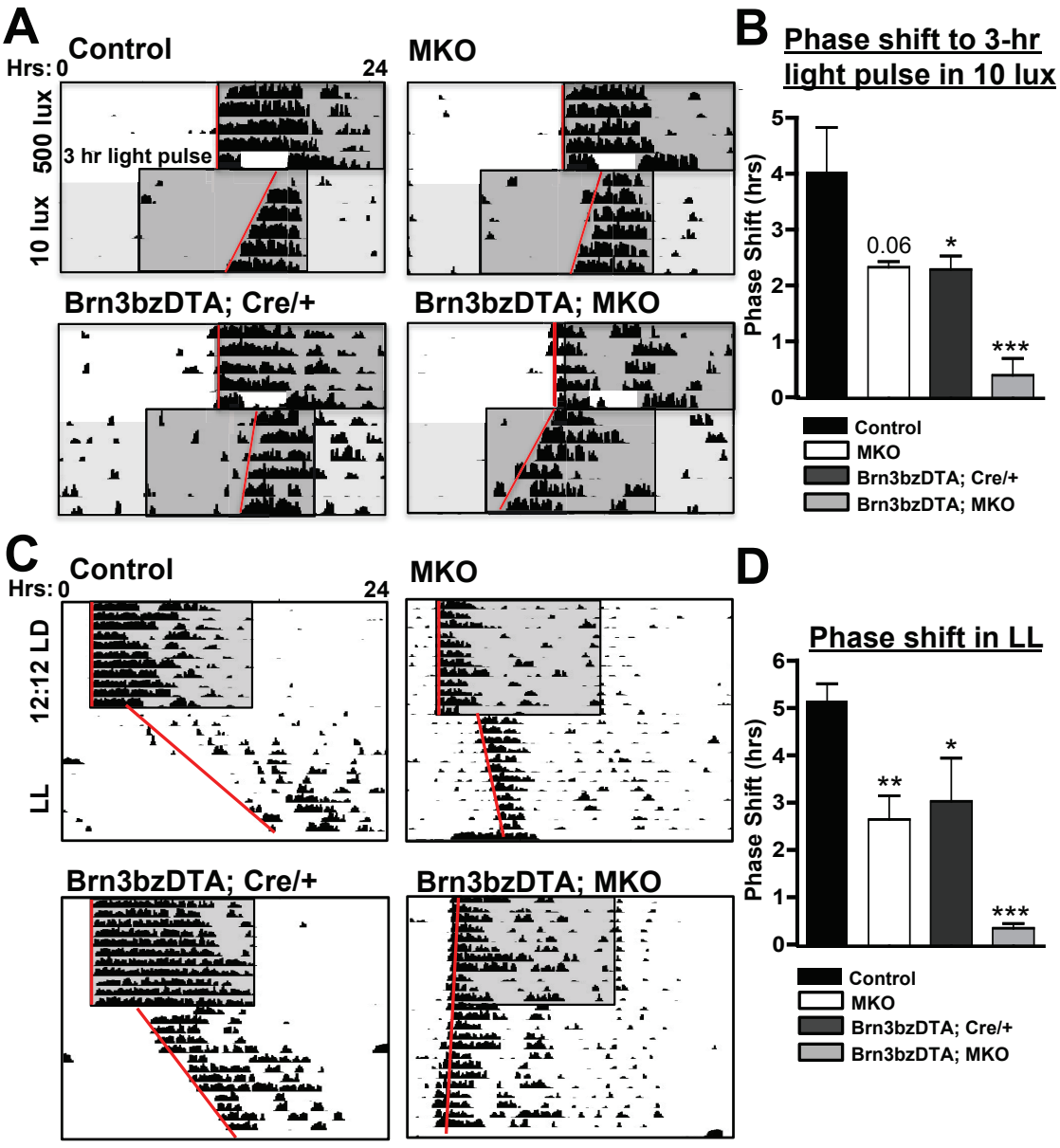


FIGURE 31: Phase shifting occurs in multiple LD environments and depends on how much the retinal circuit is intact.

A) Actograms of 500 lux 3-hr light pulse at ZT14 in a 12:12 LD cycle immediately followed by 6-hr phase advance 12:12 LD at 10 lux. B) Quantification of the phase shift in hours. C) Actograms of 500 lux 12:12 LD cycle followed LL also at 500 lux. D) Quantification of the phase shift in hours. Statistical analyses: two-tailed student's t-test. P-values: *P<0.05, **P<0.01, ***P<0.001.

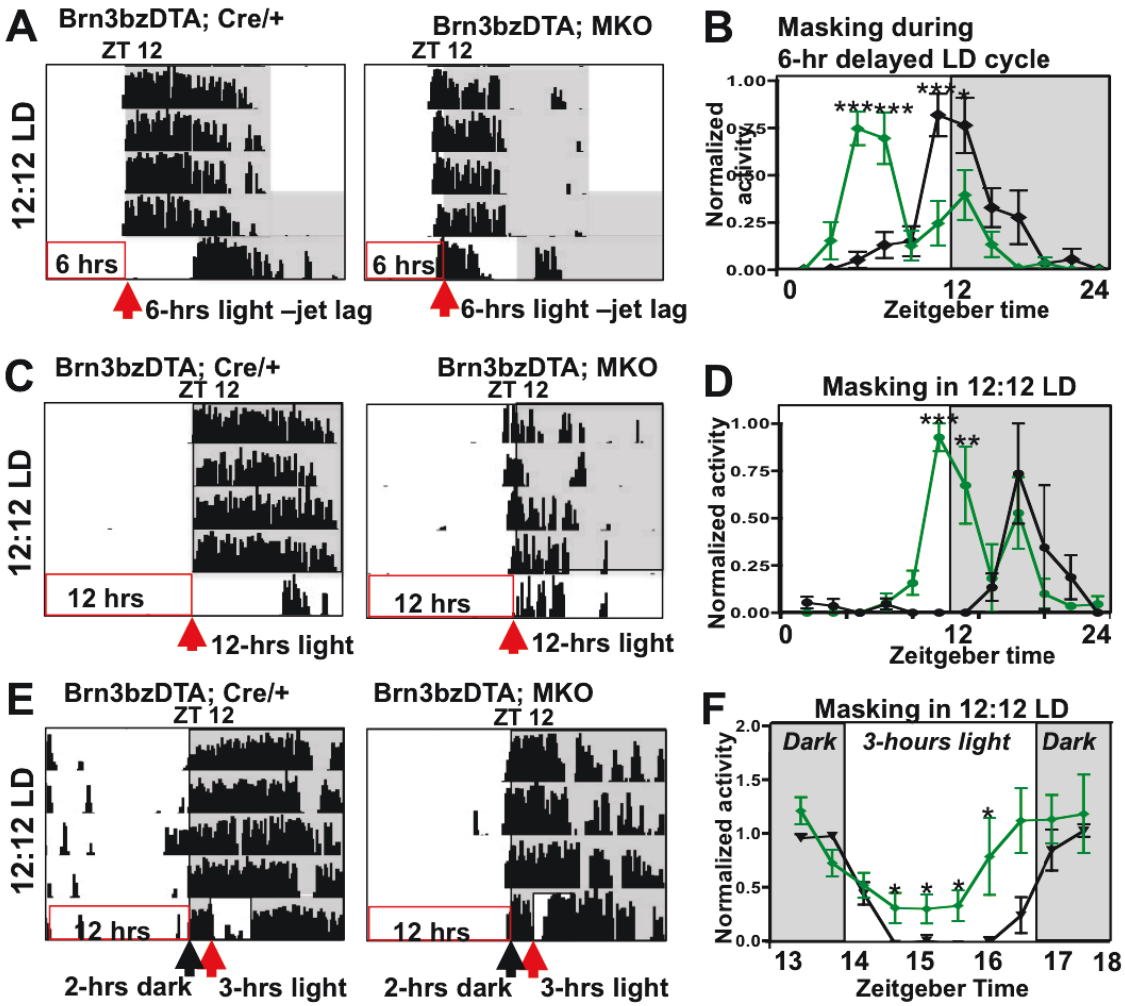


FIGURE 32: Masking requires melanopsin in SCN-projecting Brn3b-negtive ipRGCs in 12:12 LD when Brn3b-positive ipRGCs are ablated.

A) Masking on the first day in the 6-hr delayed 12:12 LD cycle in Brn3bzDTA; Cre/+ and Brn3bzDTA; MKO animals with 6 hours of light exposure before time of masking (indicated by red arrow). B) Quantification of masking on the first day of the 6-hr delayed LD cycle. ZT12 refers to the onset of darkness in the 6-hr delayed LD cycle. C) Masking in 12:12 LD in Brn3bzDTA; Cre/+ and Brn3bzDTA; MKO animals following 12 hours of light exposure before time of masking (indicated by red arrow). D) Quantification of masking during 12:12 LD. E) 3-hr light exposure at ZT 14 - ZT 17 during the 12:12 LD cycle in Brn3bzDTA; Cre/+ and Brn3bzDTA; MKO animals. F) Quantification of activity before, during and after 3-hr light exposure at ZT 14 - ZT 17. Activity graphed in 30-minute bins and normalized to activity one-hour prior to light pulse for each animal. Statistical analyses: two-tailed student's t-test. P-values: *P<0.05, **P<0.01, ***P<0.001.

Melanopsin in Brn3b-negative ipRGCs is necessary for circadian photoentrainment during 16:8 LD cycle.

To answer this question, I placed Brn3bzDTA; Cre/+ animals in 16:8 LD and subjected them to the same manipulations of the LD dark environment used in my previous experiments in 16:8 LD. I found that like control animals, Brn3bzDTA; Cre/+ animals photoentrain in 16:8 LD with a phase angle of entrainment and onset stability of activity in 16:8 LD is both similar to control and significantly different from MKO animals (Figure 33 A, B, and C). Importantly, during 16:8 LD, Brn3bzDTA; Cre/+ animals re-entrain to the 6-hrs shifted jet lag paradigm in a similar manner to Control animals (Figure 33 A and D). In the absence of melanopsin in Brn3b-negative ipRGCs, I observed major deficits in circadian photoentrainment in the 16:8 LD jet lag paradigm. These data indicate that the presence of melanopsin in the Brn3b-negative ipRGCs is necessary and sufficient, along with extrinsic rod/cone signaling, for circadian photoentrainment in both 12:12 LD cycle and 16:8 LD cycle.

Next, I wanted to determine if the masking and phase shifting profile in 16:8 LD for the Brn3bzDTA; Cre/+ animals are comparable to the control animals. Therefore, I analyzed the whether Brn3bzDTA; Cre/+ animals exhibited the partial masking phenotype observed in control animals during the first day of the 6-hr delayed 16:8 LD. Similar to

control animals, I found that Brn3bzDTA; Cre/+ animals exhibited either no masking or complete masking in a close to 50:50 ratio (Figure 34). Finally, I found that Brn3bzDTA; Cre/+ animals mask their activity to 3-hrs of light during the early night (ZT14-17) but exhibit no phase shifting (Figure 35). Together, my data suggests that presence of melanopsin in control animals is important for the regulation of masking during 16:8 LD cycle.

If masking contributes to photoentrainment during the 6-hr delayed 16:8 LD cycle, then enhancing the masking ability in MKO animals should help them re-entrain in fewer days in the 6-hr delayed 16:8 LD cycle (see fig 24A-D). In order to enhance the masking ability of MKO in 16:8 LD, I exposed mice to 2-hrs of darkness on the first two days in the 6-hr phase delay. The 2-hrs of darkness was at the time just prior to novel 6-hrs of light exposure in the delayed 16:8 LD cycle, when WT mice would normal exhibit masking. I predicted that 2-hrs of darkness would be sufficient to dark-adapted rods and cones, enabling them to drive a stronger light-mediated masking response. I used two days of darkness in order to find a medium between an acute exposure (one day) and chronic (the entire time of re-entrainment, 3-5 days). I found that 2-hrs of darkness was sufficient to allow MKO animals to mask their activity to light during the 6-hr delayed 16:8 LD cycle (Figure 36 A). The rate of re-entrainment with the 2-hrs of darkness produced a significant reduction of the number of days for MKO animals to re-

entrain in 16:8 LD (Figure 36 A and B). These data strongly support my conclusion that masking, in addition to phase shifting, plays a key role in photoentrainment during the jet lag paradigm.

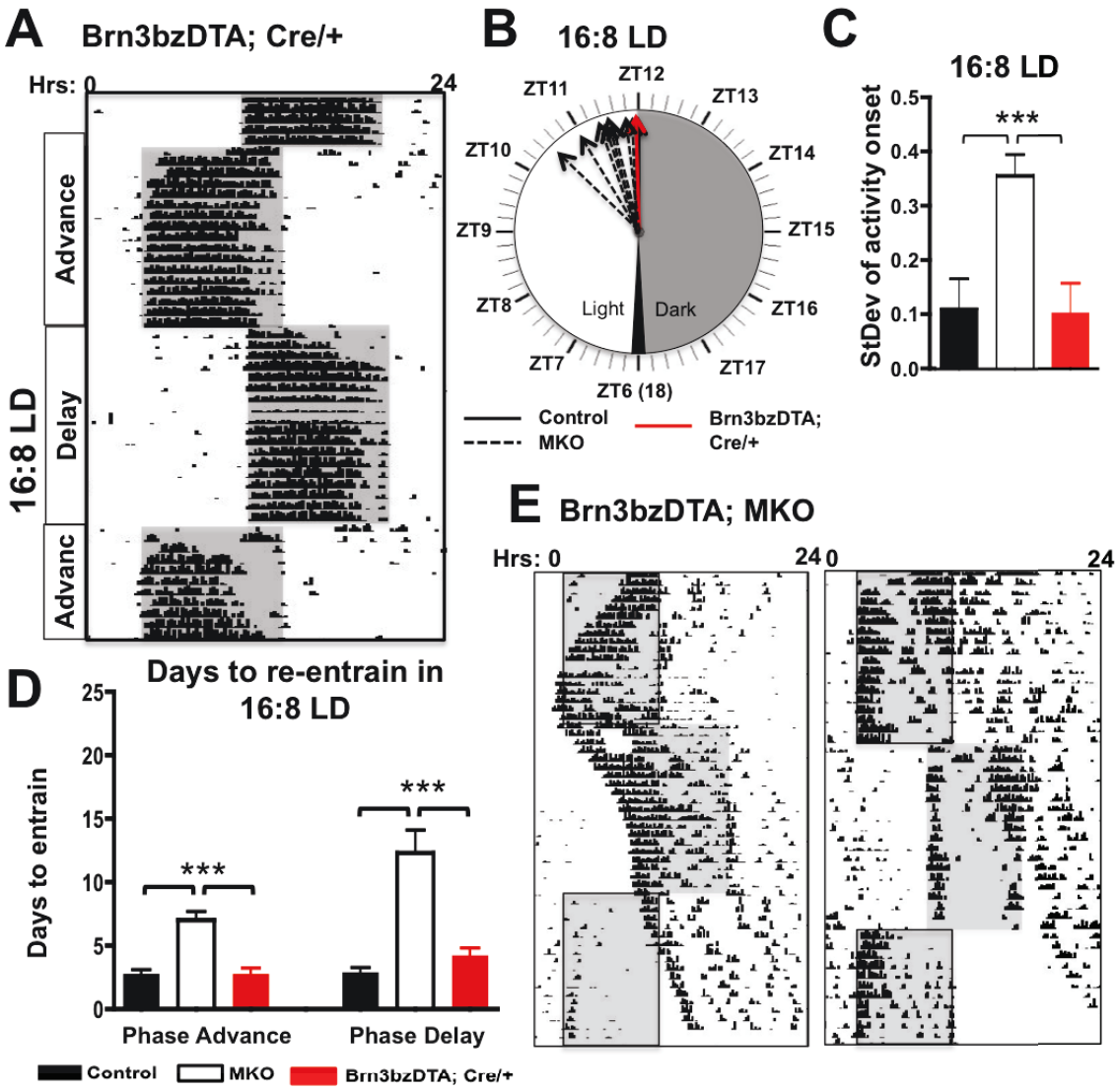


FIGURE 33: In the presence of melanopsin, Brn3b-negative ipRGCs are sufficient to drive WT-like photoentrainment and re-entrainment in the 16:8 LD cycle.

A) Re-entrainment to 6-hr phase advance, 6-hr phase delay, and 6-hr phase advance in a 16:8 LD for Brn3bzDTA; Cre/+ animals at 500 lux. B) Phase angle of alignment for Control, MKO and Brn3bzDTA; Cre/+ 16:8 LD cycle. Average alignment is shown to ZT12 for Control and Brn3bzDTA; Cre/ + animals. Each dashed black arrow represents activity onset for a single MKO mouse. C) Stability of activity onset in 16:8 LD cycle for Control, MKO and Brn3bzDTA; Cre/+ animals; measured by the standard deviation in activity onset over 10 days. D) Quantification of number of days to re-entrain to the 6-hr phase advances and delay during the 16:8 LD cycle. E) Re-entrainment to a to 6-hr phase advance, 6-hr phase delay, and 6-hr phase advance in a 16:8 LD for Brn3bzDTA; MKO animals at 500 lux. All Control and MKO data is re-plotted from figures 22 and 23. Statistical analyses: two-tailed student's t-test. P-values: ***P<0.001.

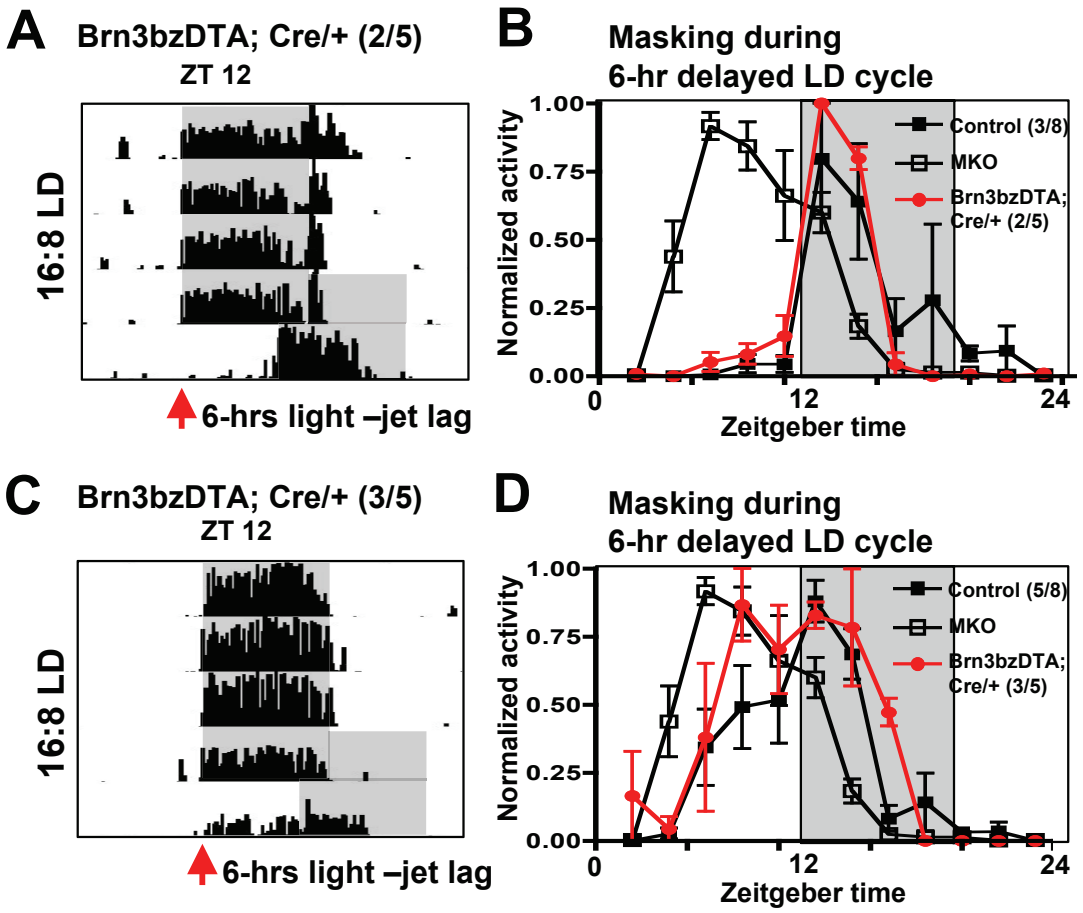


FIGURE 34: Masking on the first day in the delayed 16:8 LD cycle is partially disrupted in the Brn3bzDTA; Cre/+ animal in a WT-like manner.
A) Actogram of masking on the first day in the delayed 16:8 LD cycle--- 2/5 animals show this phenotype. B) Quantification of (A) graphed in 2 hour bins of activity. C) Actogram of masking on the first day in the delayed 16:8 LD cycle--- 3/5 animals show this phenotype. D) Quantification of (C) graphed in 2 hour bins of activity. All Control and MKO data is re-plotted from figure 24.

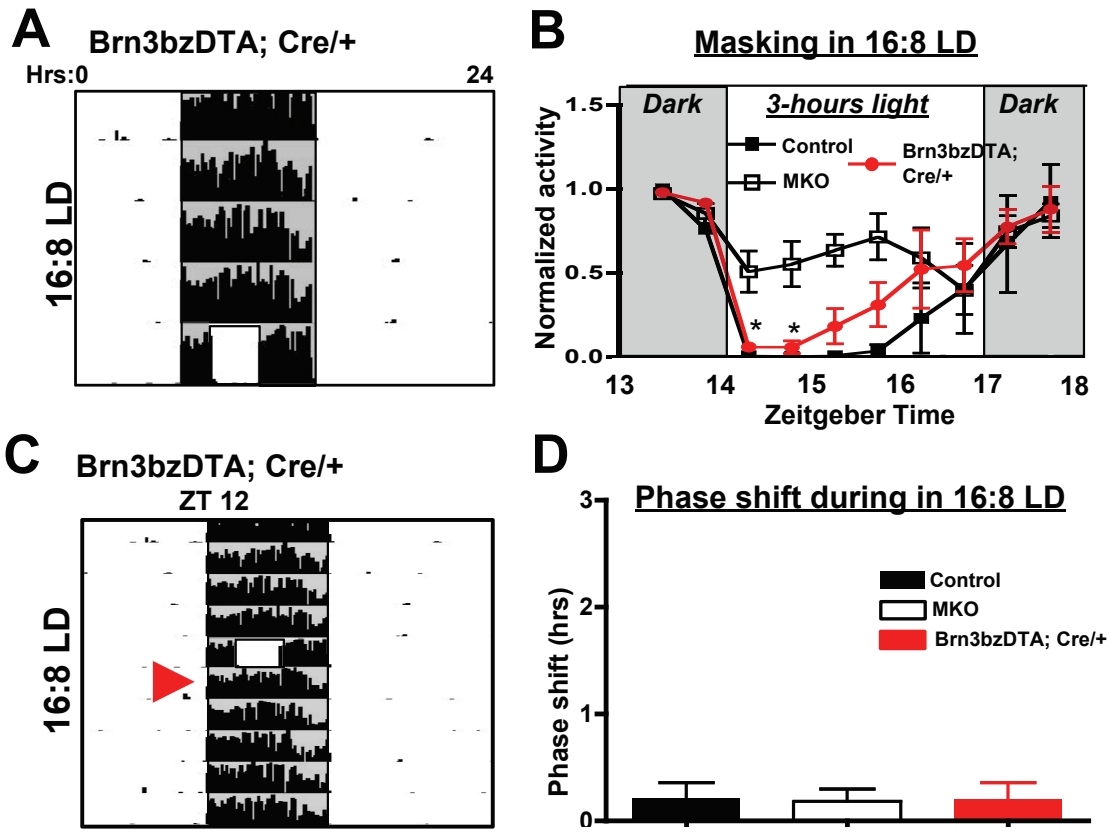


FIGURE 35: During 16:8 LD, Brn3bzDTA; Cre/+ animals exhibit masking to 3-hrs of light during the early night but do not phase shift.
A) Masking to 3-hr light exposure at ZT 14 - ZT 17 during the 16:8 LD cycle in Brn3bzDTA; Cre/+ animal. B) Quantification of activity before, during and after 3-hr light exposure at ZT 14 - ZT 17. Activity graphed in 30-minute bins and normalized to activity one-hour prior to light pulse for each animal. C) Phase shift induced by 3-hr light exposure at ZT 14 - ZT 17 during the 16:8 LD cycle in Brn3bzDTA; Cre/+ animal. First day of shift indicated with red arrowhead. D) Quantification of phase shift induced by 3-hr light exposure at ZT 14 – ZT 17 during the 16:8 LD cycle. All Control and MKO data is re-plotted from figures 25 and 26. Statistical analyses: one-way ANOVA. P-value: *P<0.05.

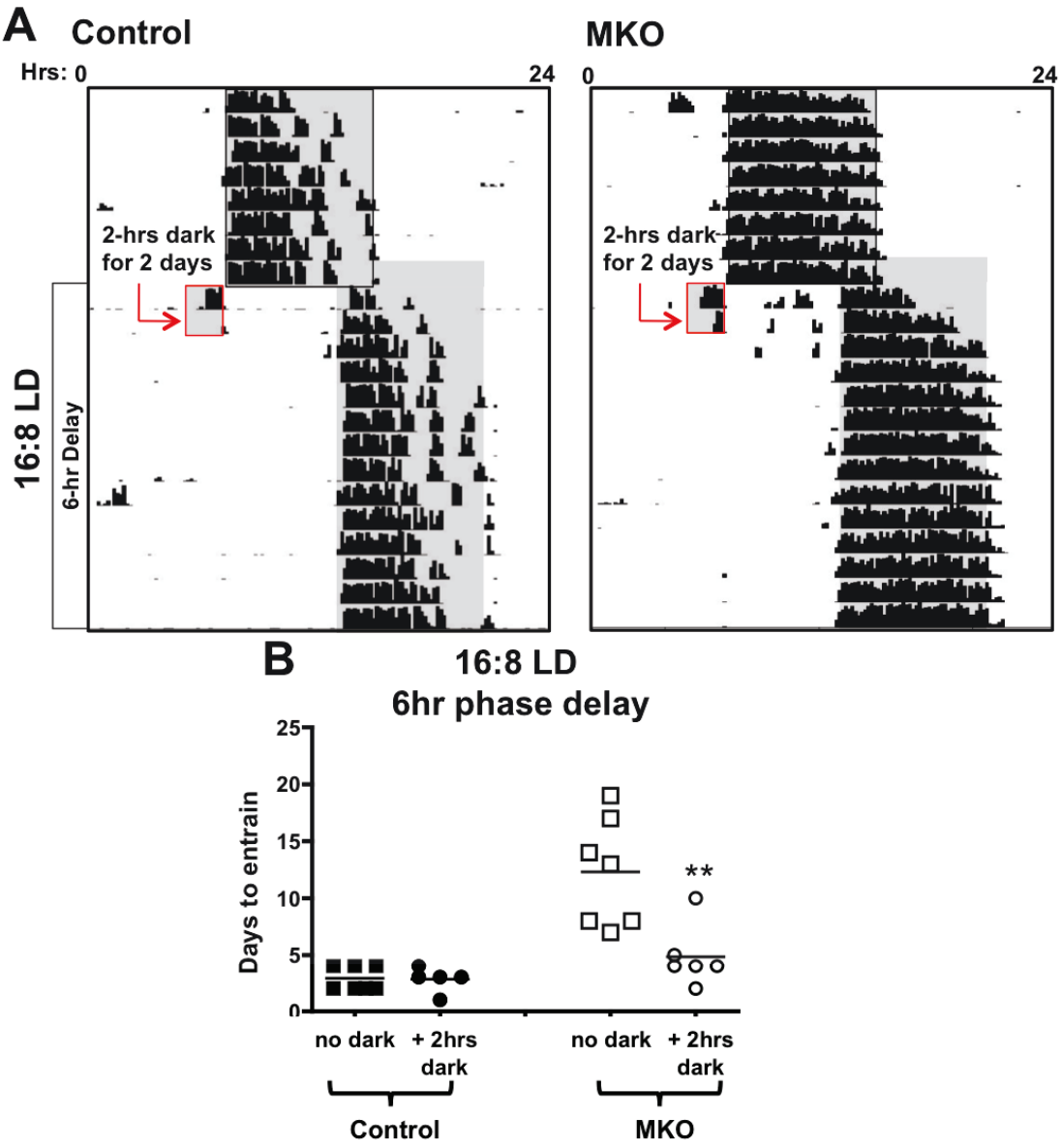


FIGURE 36: 2-hours of darkness on the first two days in the 6-hr delayed 16:8 LD cycle rescues masking deficit and increases the speed of re-entrainment in the absence of melanopsin.

A) Actograms depicting re-entrainment to 6-hr delayed 16:8 LD cycle with 2-hrs dark exposure on the first two days in delayed LD cycle in control and MKO animals. B) Quantification of the number of days to re-entrain to the 6-hr delayed 16:8 LD cycle with the dark exposure in control and MKO animals. "No dark" Control and MKO data is re-plotted from figures 23. Statistical analyses: two-tailed student's t-test. P-value: **P<0.01.

DISCUSSION AND CONCLUSIONS

In this study, I provide 4 new findings. 1) I reveal the first circadian photoentrainment deficits in MKO animals. Since the generation of these animals, several studies have concluded that circadian photoentrainment is normal in MKO animals despite circadian phase delay deficits (Panda *et al.,* 2002; Ruby *et al.,* 2002). Here, using light dark cycles with different day lengths, I uncover that MKO animals have deficits in circadian photoentrainment. 2) I uncover that brain regions distinct from the SCN contribute to circadian photoentrainment in the absence of melanopsin protein, when ipRGCs solely depend on rod/cone input. 3) I show that circadian phase advances and delays in a jet lag paradigm are not equivalent. 4) I implicate that masking deficits could lead to problems in circadian photoentrainment.

Advanced phase angle

In all the genetic mouse lines that I used and under most light conditions, animals were able to show circadian photoentrainment, by the strict definition of their period length becoming 24 hours. However, there were important differences in the phase angle of entrainment and the stability of phase onsets between different conditions. Although I found that melanopsin is generally important for normal phase alignment, control animals also exhibited an advanced phase angle during 20 and 23 hr days. Furthermore, animals that lack melanopsin

and non-SCN projecting ipRGCs, also showed advanced phase angle of entrainment and unstable onset of activity. Combined these results show that rod/cone input is sufficient for normal phase angle of entrainment, but only under defined day length conditions and when the non-SCN brain targets of ipRGCs are also innervated. This data suggests that phase angle alignment may be an indicator of other normal circadian functions, more of which are disrupted in the absence of melanopsin.

Re-entrainment in the jet lag paradigm: advances versus delays

Classically, the phase response curve was used as a proxy to explain circadian photoentrainment (Decoursey 1986). However, despite deficits in phase delays in MKO animals, these animals showed no deficits in circadian photoentrainment to light cycles that require delay in the phase of the circadian oscillator (Ruby *et al.,* 2002). In this study, I find that masking deficits correlate well with deficits in circadian photoentrainment. I was able to reveal a deficit in circadian photoentrainment in MKO animals that are maintained under 16:8 LD cycle. I revealed that this deficit is correlated with weaker masking responses in these animals. Remarkably, the masking responses were observed even in animals that received 2 hours of dark adaptation sufficient to restore full rod/cone responses in the retina. This indicates that masking responses are dependent on prior light exposure or what is

known as light history. Perhaps most surprisingly, is that re-entrainment to advances or delays of the light dark environment were not equivalent.

Non-SCN regions involved circadian functions

The fact that I observed deficits in MKO animals led us to the question of whether further deficits can be observed in MKO animals that also lack innervation to the majority of non-SCN targets (Chen *et al.,* 2011). That is to say, can I uncover an unappreciated role to non-SCN regions in circadian photoentrainment? Previously, I generated an animal line where the ipRGCs that project to non-SCN regions are predominantly eliminated (Chen *et al.,* 2011). Using these animals, I showed that under high light intensity and the presence of melanopsin, circadian photoentrainment is fully intact (Chen *et al.,* 2011). Here, I used this genetically modified mouse line, but now with the melanopsin protein eliminated as well. This led us to discover a surprising role for non-SCN projecting ipRGCs in relaying rod/cone information for masking, circadian phase alignment, and re-entrainment under 12:12 LD cycle and under high light intensities. This data indicates that brain regions other than SCN play a role circadian photoentrainment driven by rod/cone input. While ipRGC targets such as the OPN is not likely to be involved in regulating circadian functions, targets such as the intergeniculate leaflet (IGL) and supraoptic nucleus (SON) could play a

role (Hattar *et al.,* 2006). Future studies would aim to uncover the brain region(s) responsible for these effects.

I then investigated whether animals that have melanopsin but lack input to non-SCN regions would show deficits in circadian photoentrainment under light levels that are below the sensitivity of melanopsin-based photo response. Using Brn3bzDTA; Cre/ + and Brn3bzDTA; MKO animals, I found that at dim light intensities, both SCN and non-SCN projecting ipRGCs are necessary to mediate light input for circadian photoentrainment and masking. This finding indicates that the absence of melanopsin in Brn3bzDTA; MKO is not developmentally modifying Brn3b-negative ipRGC rod/cone input. Specifically, I find that at dim light, where only rods are sensitive enough to relay light information to the brain, the intrinsic melanopsin response in the Brn3bzDTA; Cre/+ can no longer drive normal photoentrainment.

Combined our studies reveal three central discoveries. First, delays and advances to shifts in the light-dark cycles are not equivalent. Second, masking may contribute to circadian photoentrainment in a day length dependent manner. Third, non-SCN regions play a critical role in circadian photoentrainment for light intensity below the sensitivity of the melanopsin phototransduction pathway. These studies provide new avenues for understanding circadian photoentrainment as well as the circuits that underlie how light through rods/cones and melanopsin

phototransduction interact to drive photoentrainment under different seasons.

THESIS CONCLUSION

Each individual uniquely senses the light-dark environment to regulate non-visual light responses such as circadian photoentrainment. In contrast to our predictable day and night cycle, there are daily variations in the light-dark environment such as: changes in day length, trans-meridian travel, varying light intensities, and differences in the timing of the exposure to any of these light stimuli. It is plausible to think that in brain, there are multiple circuits involved in responding to these variations in the light-dark environment. Circadian photoentrainment must consistently occur each day, even with such environmental variability.

During my studies, I have found that disruptions of retinal circuits lead to a failure in circadian photoentrainment and can result in period lengthening. Intrinsically photosensitive retinal ganglion cells (ipRGCs) and their brain recipient targets are likely to use multiple retinal pathways, in order to maintain a stable response to the light-dark environment when there are variations in the environment that occurs each day. I predict that the various ipRGC-subtypes may differentially interact with the rods and cones, through the retinal circuitry to specifically contribute to circadian photoentrainment, in an environment-specific manner. Not only could the retina hold the complexity of multiple interacting pathways to drive circadian photoentrainment, but also brain nuclei such as the SCN and the IGL. Sub-regions of these nuclei may

receive input about specific light information in the environment. My work has demonstrated that the brain uses multiple circuits to regulate circadian photoentrainment. This body of work sheds light on the multi-modal nature of how the retina uses light information to regulate circadian photoentrainment.

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CURRICULUM VITAE

Melissa Elizabeth Simmonds

Born: October 17, 1986

Jamaica, West Indies

EDUCATION

Ph.D., Johns Hopkins University, Department of Biology, March 2015

Advisor: Dr. Samer Hattar

"The role of melanopsin in the regulation of circadian behavior and light responses under a wide variety of light–dark environments"

B.S. Oakwood University, Department of Biological Sciences, May 2009

Major: Biology; Minor: Music, with a piano concentration

ACADEMIC AND PROFESSIONAL HONORS

2005	National Dean's List
2005	Student-athlete academic excellence awards from City College of New York
2006	GPA-based Transfer Scholarship to Oakwood University
2007-2008	Two Medals awarded for achieving the Dean's List at Oakwood University, (annual award)
2007	Alpha Chi National Honor Scholarship Society
2009	Awarded Student of the Year in the Biology Department at Oakwood University
2009	Graduated Cum laude
2009	Full tuition scholarship and living stipend for a Ph.D. in Biology at Johns Hopkins University.
2013	Carl Storm Travel Award for travel to the Gordon Conference
2013	The Carl Lokko Lecturing scholarship for Oakwood

Alumni

2014 Certificate for the acknowledgement of Mentorship from Morgan State University

2014 Society for Research on Biological Rhythms (SRBR) Research Excellence Award

GUEST LECTURES AND PROFESSIONAL INVITATIONS

Fall 2009 Lectured to the Biology department at Oakwood University on the Alternative splicing and Sexual dimorphism in Drosophila during a Johns Hopkins University recruitment visit.

Spring 2009 Taught Developmental Biology Class at Oakwood University.

Spring 2012 Lectured on my research to 3rd and 4th year undergraduates at Oakwood University.

Spring 2012 Lectured on the sensory nervous system and my research to Biology and nursing students at Northern Caribbean University, Jamaica.

Fall 2013 Lectured on my research at the Oakwood Biological and Medical Association meeting at Oakwood University.

Fall 2013 Invited to Morgan State University as a panelist to discuss “how to succeed in graduate school”.

Fall 2013 Seminar speaker for Morgan State University RISE program seminar series.

Spring 2014 Invited to Morgan State University as a Mock Interviewer for graduate school-bound junior and senior undergraduates.

Summer 2014 Session speaker at the Society for Research on Biological Rhythms in Big Sky, MT

ABSTRACTS AND PRESENTATIONS

Year	Presentation Type	Event/Institution
2007	Poster	Summer Students Poster session Johns Hopkins University
2007	Poster	Annual Bio-Symposium at Oakwood University
2007	Poster	Annual Bio Retreat at University of Alabama in Huntsville
2007	Abstract	ABRCMS
2008	Poster	Johns Hopkins University
2012	Poster/Abstract	ABRCMS
2012	Poster/Abstract	Greater Baltimore Chapter of the Society for Neuroscience
2013	Poster/Abstract	ARVO (Association for Research in Vision and Opthalmology)
2013	Poster/Abstract	Gordon Research Conference (Chronobiology)

POSITIONS

ACTIVITY/OCCUPATION	DATE	FIELD	INSTITUTION
Undergraduate summer researcher (SURE Program)	May- Aug 2007	Neuro-science	Johns Hopkins University
Developmental Biology Lab Instructor	Jan- May 2008	Biology	Oakwood University
RISE-I-CARE undergraduate researcher	Fall 2007- Spring 2008	Biology	Oakwood University
Undergraduate summer researcher (SURE Program)	Summer 2008	Neuro-science	Johns Hopkins University
Histology Lab Instructor	Fall 2008 to Spring	Biology	Oakwood University

	2009		
SURE team leader and mentor	Summer 2010	Biology	Johns Hopkins University
Graduate program recruiter at ABRCMS	Nov 2010	Biology	Johns Hopkins University
Biochemistry Lab Instructor	Fall 2010	Biochemistry	Johns Hopkins University
Development Biology Lab Instructor	Spring 2011	Biology	Johns Hopkins University
SURE student mentor	Summer 2011	Biology	Johns Hopkins University
Poster session judge at ABRCMS	Nov 2011	Neuroscience	ABRCMS
Graduate program recruiter at ABRCMS	Nov 2011	Biology	Johns Hopkins University
Poster session judge at ABRCMS	Nov 2012	Neuroscience	ABRCMS
Graduate program recruiter at ABRCMS	Nov 2012	Biology	Johns Hopkins University
Life, Planets, and Universe Teacher's Assistant	Fall 2012	Astrobiology	Johns Hopkins University
Emerging Strategies in Biology Teacher's Assistant	Spring 2013	Biology	Johns Hopkins University
Genetics Teacher's Assistant	Fall 2013 - Current	Biology	Johns Hopkins University
Online multiple-subject tutor	July 2012 - Current	Biology, music, life sciences	InstaEDU
Intro to the Human Brain Teacher's Assistant	Spring 2014	Biology	Johns Hopkins University
Graduate program recruiter and poster session judge at ABRCMS	Nov 2014	Biology	Johns Hopkins University

Human Genetics Teacher's Assistant	Fall 2014	Biology	Johns Hopkins University

Appendix: Investigation of ligands and receptors potentially involved in SCN innervation by ipRGCs.

INTRODUCTION

During development of the visual system, axons of retinal ganglion cells (RGCs) in mammals approach the optic chiasm and partially decussate to form the optic tracts (Godement *et al.*, 1987; Colello and Guillery, 1990; Godement *et al.*, 1990; Sretavan and Reichardt, 1993; Marcus and Mason, 1995). RGCs decussation is regulated by axon guidance cues expressed in the surrounding cells of the ventral hypothalamus. Axon guidance cues in the ventral hypothalamus, such as slits, ephrins, and semaphorins, interact with receptors on RGC axons, such as robos, ephs and plexins, to guide RGCs to their targets. Ligand-receptor pairs guide RGCs to their target through a combination of attractive and repulsive cues (Erskine *et al.*, 2000; Nakagawa *et al.*, 2000). The importance of the temporally regulated expression of ligand-receptor pairs is observed in axons that cross the midline in both vertebrates and invertebrates (Fricke *et al.*, 2001; Plump *et al.*, 2002). These and other studies of RGC axon guidance has led to a much better understand of how the visual system functions.

There is much unknown about how the RGCs that target the SCN to drive circadian photoentrainment (ipRGCs) innervate the SCN. Understanding more about this process could shed more light on how the retina interacts with the SCN to drive circadian photoentrainment. Compared to the number of RGCs involved in vision, ipRGCs that

innervate the SCN are few in number and diverge from the majority of RGCs and ipRGCs, the axons of which enter the optic tract to innervate brain areas for vision and other non-image forming functions. ipRGCs innervate the SCN, which is located dorsal to the optic chiasm, where the optic nerve axons decussate to form the optic tracts. Axons of ipRGCs arrive at the optic chiasm just outside the SCN at embryonic day 17 (E17), but do not enter the SCN until P1 (McNeill *et al.*, 2011). It is not understood how this small population of axons leave the optic chiasm to target the SCN in order to mediate the essential task of circadian photoentrainment.

RESULTS

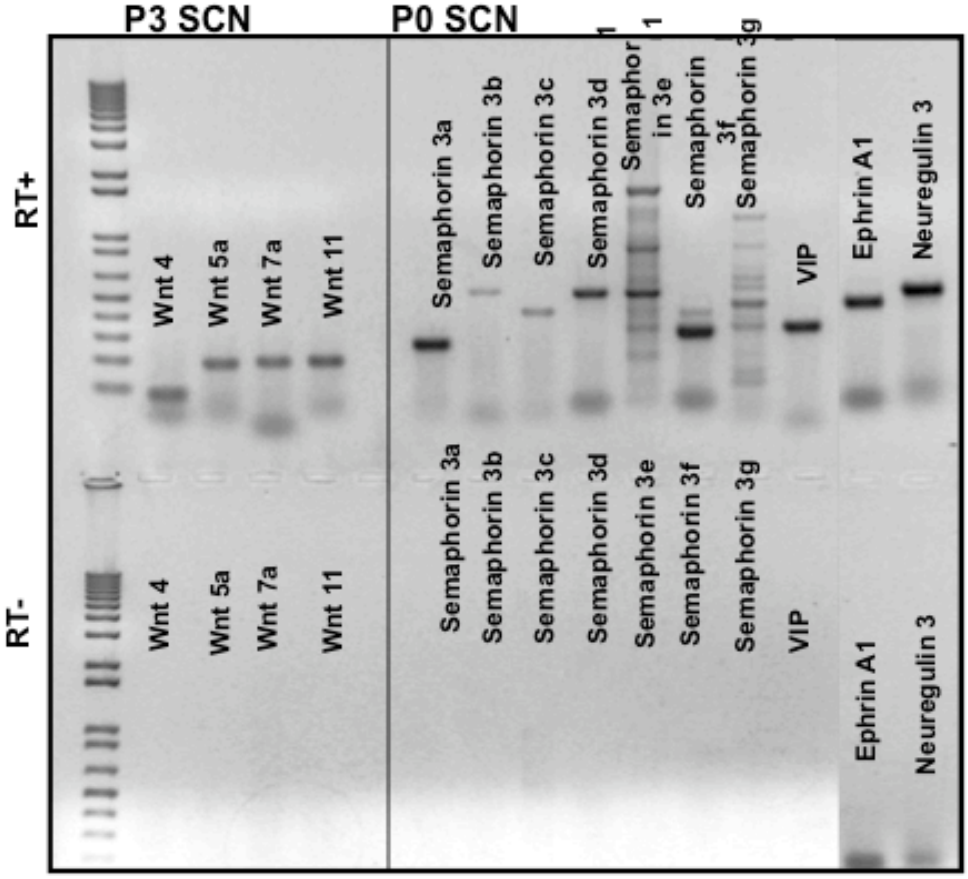
The delay in the SCN innervation from ipRGCs could be due to a repulsive signal, the lack of an attractive signal, or both. The SCN is a functional nucleus embryonically, and therefore, between E17 and P1, it is unlikely that a repulsive cue from the SCN would repel ipRGC axons and delay SCN innervation. Therefore, I conducted an RT-PCR screen with the following prediction: An attractive axon guidance cue is expressed in the SCN at P1 when ipRGC axons start the innervation of the SCN. Template cDNAs for the RT-PCR screen were generated from tissue of the ventral hypothalamus that included the SCN and nearby surrounding tissue at P0 and P3. The screen identified 19 of the 25

ligands assayed, including Ephrin A1 (Appendix Figure 1; 14 of the positively identified ligands shown). Due to their involvement in axon guidance of conventional RGCs at the optic chiasm, many candidates from the RT-PCR screen were eliminated from further investigation.

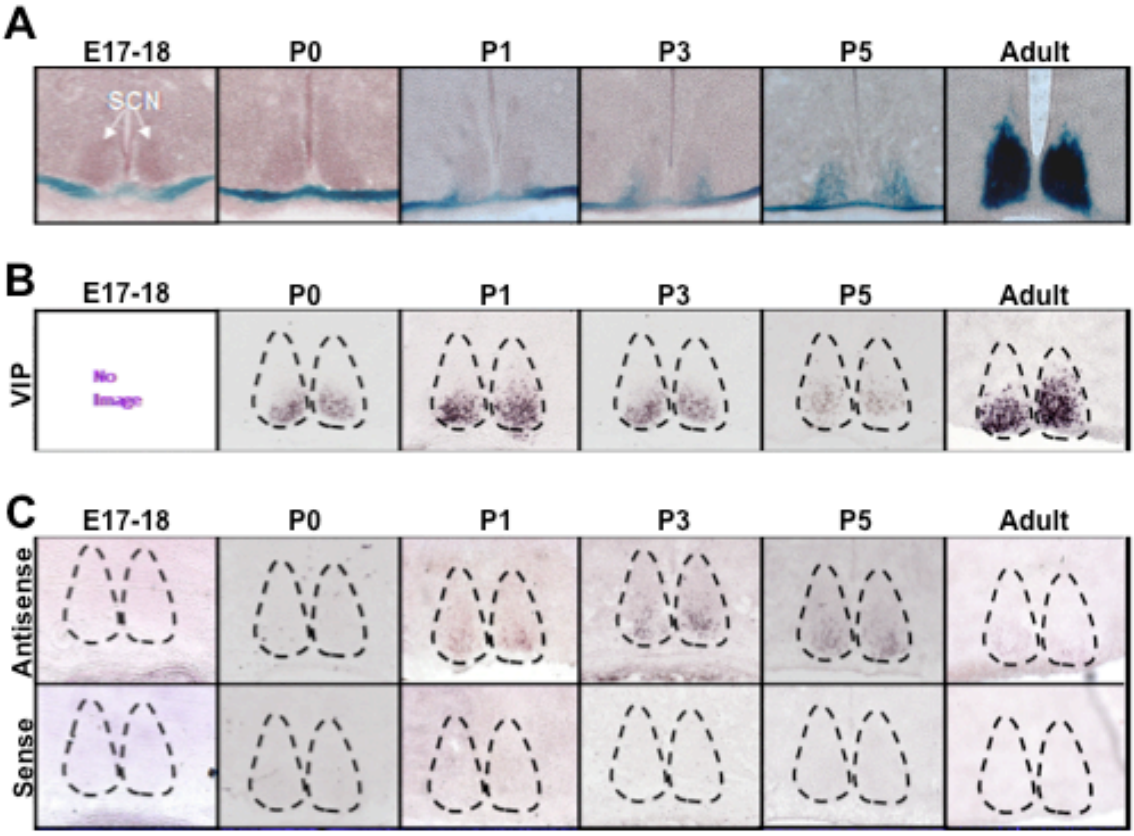
I successfully developed riboprobes for 6 ligands from the RT-PCR screen: Semaphorin 3a, Semaphporin 3c, Semaphorin 3d, Neuregulin 3, Wnt5a, and Ephrin A1. I then performed *in situ* hybridization on E17-18, P0, P1, P3 and adult SCN tissue to determine if these ligands were expressed in the SCN during the time ipRGCs innervate the SCN. The *in situ* screen led to the successful identification of only one ligand in the SCN, Ephrin A1. The expression of Ephrin A1 by *in situ* coincided with the innervation pattern of the SCN. These data show that prior to Ephrin A1 expression, the SCN is not innervated; however, as Ephrin A1 expression in the SCN is detected via *in situ* hybridization (at P1), ipRGC axons begin innervation to the SCN (Appendix Figure 2). These findings are consistent with a role played by Ephrin A1 in the axon guidance of ipRGCs to the SCN during development.

To determine whether the temporal protein expression pattern of Ephrin A1 would be similar to that of the mRNA expression, I performed analysis of protein in the SCN both an immuno staining and Western blot. Ephrin A1 protein levels are higher following SCN innervation at P3 and in adult than they are before innervation at P0 (Appendix Figure 3).

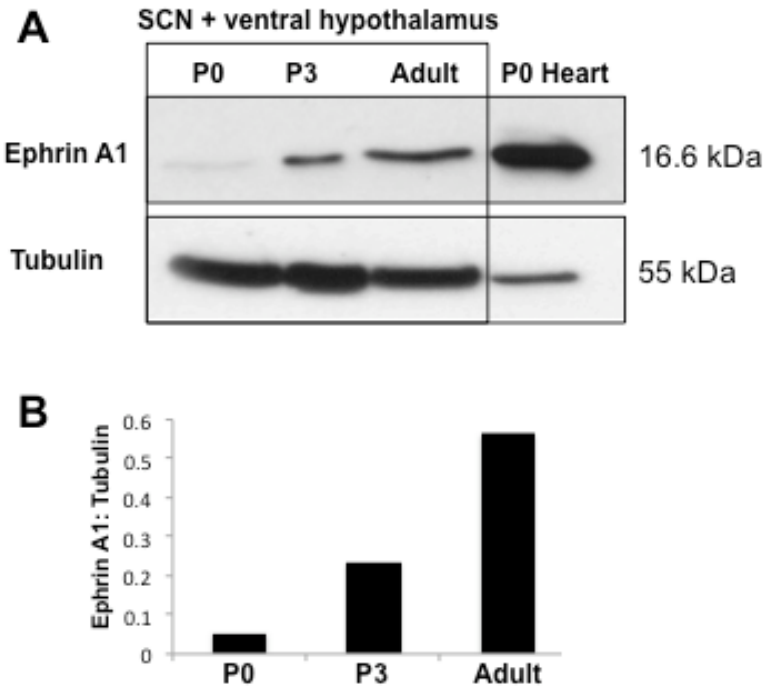
B-actin was used as a control for protein level. The attractive interaction between EphA3 and Ephrin A1, in neighboring cells of the atrioventricular endocardial cushions, is necessary for the epithelial to mesenchymal transformation that occurs during heart development (Chen 2011 and Stephen et al 2006). Therefore I used heart tissue as a positive indicator of Ephrin A1 expression. These data provide a nice correlation of the temporal protein expression levels with both the spatial and temporal expression of Ephrin A1 mRNA in the SCN.



APPENDIX FIGURE 1: RT-PCR screen for axon guidance ligands in the SCN.
Screen was conducted at P0 and P3. VIP is the control for SCN tissue. RT- is the negative control for each primer tested.



APPENDIX FIGURE 2: Ephrin A1 is expression in the SCN coincides with the ipRGC innervation from the retina.
A) M1 ipRGCs labeled with melanopsin-tau LacZ marker (blue staining) (McNeill *et al* 2011). B) *In situ* of VIP in the SCN at E17-18, P0, P1, P3 P5 and Adult. C) *In situ* of Ephrin A1 in the SCN at E17-18, P0, P1, P3 P5 and Adult. Antisense and Sense negative control shown.

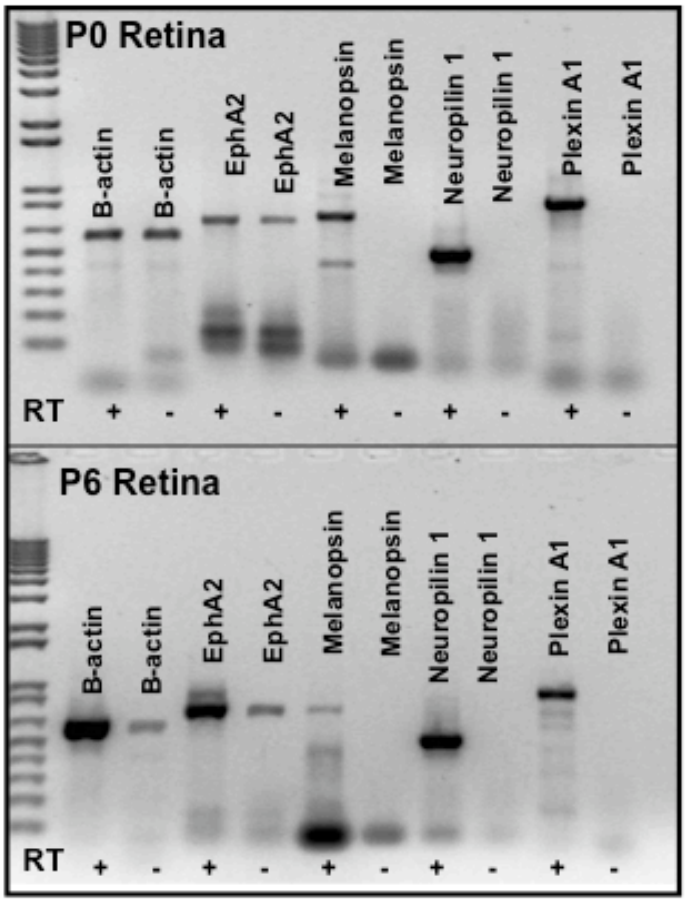


APPENDIX FIGURE 3: Ephrin A1 protein is present in P3 and adult ventral hypothalamus.
A) Western blot of Ephrin A1 and Tubulin on tissue from the SCN with the immediate ventral hypothalamus and heart in P0, P3, and Adult mice. B) Quantification of Ephrin A1 protein level in comparison to tubulin at P0, P3, and Adult mice.

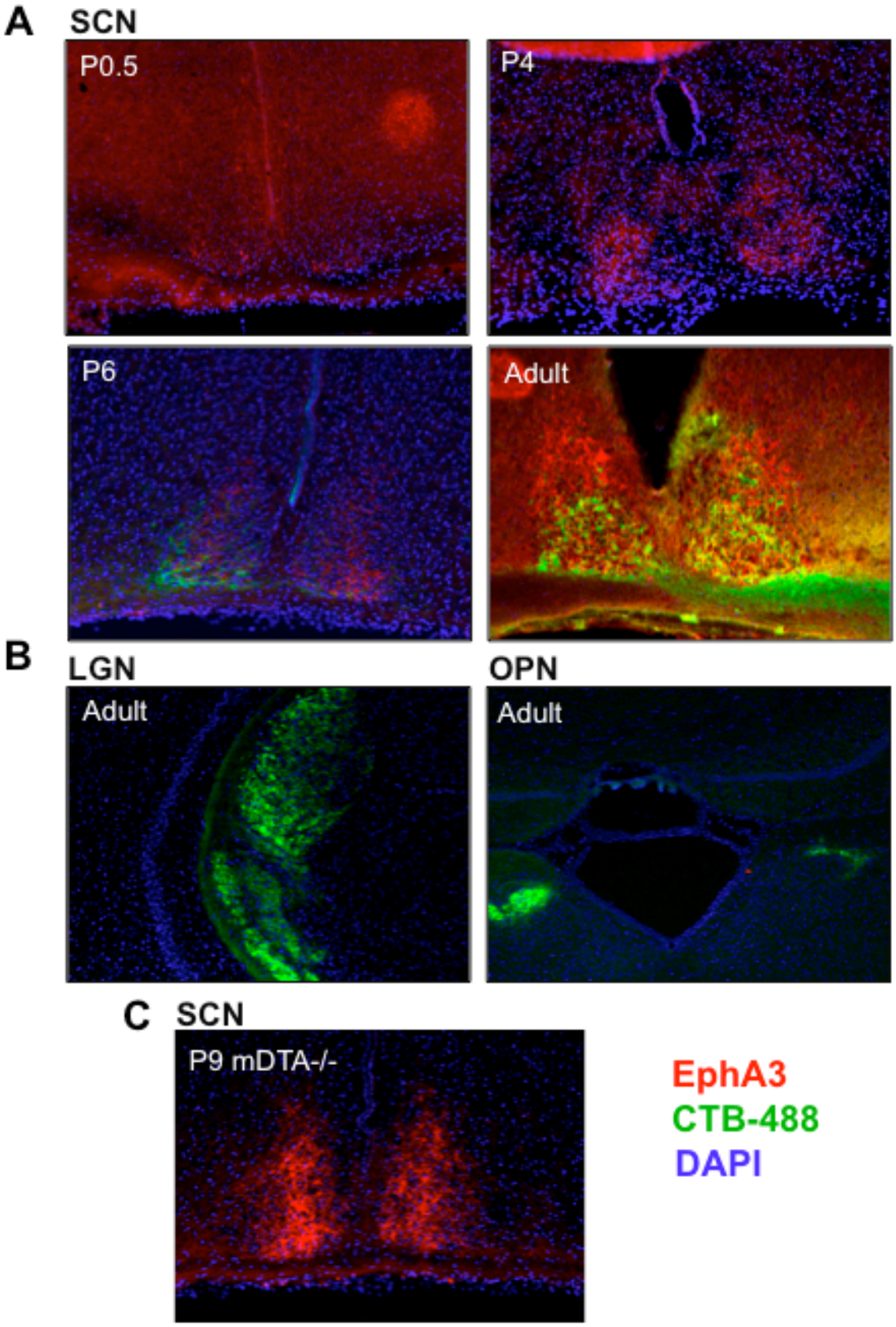
The ability of Ephrin A1 to act as either an attractive or repulsive cue depends on the interaction with its cognate receptors. Two cognate receptors for Ephrin A1, EphA2 and EphA3, have been well characterized. Research demonstrates a repulsive role for EphA2 has a repulsive role in retinal neovascularization with Ephrin A1 as its ligand (Naruse-Nakajima et al., 2001; Ojima et al., 2006; Shen et al., 2007). In an RT-PCR screen of the retina at P0 and P6, I found that EphA2 appears to have a higher expression at P6 than at P0 (Appendix Figure 4).

I was able to obtain the EphA3 antibody and investigated whether EphA3 was expressed in the axon terminals of ipRGCs in the SCN. To identify ipRGC axons, I injected cholera toxin B-488 (CTB-488) in the eyes of mice. For EphA3 to be involved in axon guidance of ipRGCs into the SCN, both it and CTB-488 would need to co-localize in the ipRGC axons, as they innervate the SCN. I found that EphA3 was expressed in a spatial-temporal manner similar to the expression of Ephrin A1 (Appendix Figure 5A). However, I noticed little co-localization with the axons of ipRGCs labeled with CTB-488 (Appendix Figure 5A). I did observe however, that EphA3 was not expressed in the OPN or IGL, two other targets of ipRGCs (Appendix Figure 5B). To further determine if EphA3 truly was expressed in the SCN and not on ipRGC axons, I

investigated the expression of EphA3 in the mDTA$^{-/-}$ animal. In the mDTA$^{-/-}$ animal, all ipRGCs are ablated. EphA3 is expressed in the SCN (Appendix Figure 5C). Therefore, EphA3 is not the expressed on the axons on ipRGCs targeting the SCN, and may not be involved in their innervation of the SCN during development.



APPENDIX FIGURE 4: RT-PCR screen for axon guidance receptors in the retina.
Screen was conducted at P0 and P6. B-actin is the control for RT reaction. RT- is the negative control for each primer tested.


A
SCN
P0.5
P4
P6
Adult
B
LGN
Adult
OPN
Adult
C
SCN
P9 mDTA-/-
EphA3
CTB-488
DAPI

APPENDIX FIGURE 5: EphA3 receptor is expressed in the SCN.
A) Immunohistochemical analysis of EphA3 in the SCN at P0.5, P4, P6 and adult. SCN co-stained for DAPI of WT animals. Cholera toxin- B-488 (CTB-488) was injected into the retina and labels RGC axons green. B) EphA3, CTB-488 and DAPI expression in the LGN and OPN of adult WT animals. C) EphA3, CTB-488 and DAPI expression in the SCN of P9 mDTA$^{-/-}$ animals.

DISCUSSION AND CONCLUSION

Identifying the temporal and spatial expression of Ephrin A1 in the SCN is the first step towards characterizing the axon guidance cues involved in ipRGC innervation of the SCN. While Ephrin A1 remains a strong candidate for an attractive cue in SCN innervation, the function of Ephrin A1 in SCN innervation remains to be determined. In my RT-PCR screen, I found many potential candidates that should be further studied. As for axonal receptors on ipRGCs that may be involved, EphA2 remains a strong candidate, as it is a known receptor of Ephrin A1 (Naruse-Nakajima et al., 2001; Ojima et al., 2006; Shen et al., 2007).

There are other receptors from my screen that may be involved in SCN innervation. One possible way to approach this study is to use the mDTA$^{-/-}$ mouse and a WT mouse, isolate the SCN, and do a comparative proteomic screen between the two tissues. The idea is that since the ipRGC axons are missing in the mDTA$^{-/-}$ mouse, any differential expression of axonal guidance cues would be strong candidates for their involvement in SCN innervation. Further understanding the how SCN innervation occurs will not only broaden our understanding of how ipRGCs target and innervate the SCN, but also how cells of seemingly similar properties can interact with such a broad variety brain regions to drive completely different functions.